

Functional analysis of Pso2 reveals a novel
DNA hairpin endonuclease activity:
Implications for interstrand crosslink repair

By

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Abstract

DNA interstrand crosslinks (ICLs) provide a challenge for repair machinery given that both strands contain the lesion. Cells have evolved a sophisticated mechanism to overcome this, by recruiting proteins from several DNA repair pathways. One protein thought to function solely in interstrand crosslinking repair is Pso2 (SNM1A in mammals). Pso2 deficient cells display sensitivity to a wide range of ICL agents and accumulate DNA double strand breaks upon exposure to these ICL chemicals. However, Pso2 is not required for repair of DNA double strand breaks generated by other means, suggesting that these particular double strand breaks are unique and require Pso2 processing prior to successful repair. To identify what characteristics these breaks possess and what role Pso2 plays in processing these breaks, a thorough *in vivo* and *in vitro* characterization of Pso2 was conducted.

Pso2 was found to be a 5'-exonuclease independent of DNA structure and length but completely dependent on the presence of a 5'-phosphate. Pso2 also displayed structure specific DNA hairpin opening activity at the 3' end two nucleotides from the apex. This activity was required for repair of genomic DNA capped by hairpin structures in the absence of ICL inducing agents as well as hairpin structures generated in response to ICL damage. Nuclease activity and *in vivo* hairpin opening required the highly conserved β -CASP domain, however, this domain on its own was not sufficient. The constitutively active DNA hairpin endonuclease β -CASP domain of Artemis was able to partially restore the DNA

hairpin opening deficiency as well as suppress the ICL defect in a *pso2* null strain. This suggests that Pso2 acts as an endonuclease in ICL repair and that DNA hairpins may be an encountered intermediate, leading to further understanding of how this unique protein function in ICL repair as well as the repair mechanism itself.

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List of Abbreviations

ATP – Adenosine triphosphate
bp – base pair
ds – double strand
DNA – Deoxyribose nucleic acid
DSB – Double strand break
DTT - Dithiolthreitol
EDTA – Ethylenediaminetetraacetic acid
EMSA – electrophoretic mobility shift assay
GST – Glutathione S transferase
HPC – Hydrogen peroxide conjugated
ICL – Interstrand crosslink
IPTG – Isopropyl β -D-1-thiogalactopyranoside
IR – ionizing radiation
HRR – Homologous recombination repair
LDAO - Lauryldimethylamine-oxide
MMC – Mitomycin C
NER – Nucleotide excision repair
NHEJ – Non-homologous end joining
nt – nucleotide
PAGE – Polyacrylamide gel electrophoreses
PCNA – Poliferating cell nuclear antigen
PCR – polymerase chain reaction
PMSF - phenylmethanesulfonylfluoride
PNKP – Polynucleotide Kinase/Phosphatase
PVDF - Polyvinylidene fluoride
RFC – Replication factor C
RNA – ribose nucleic acid
RPA – Replication protein A
SDS – sodium doceecylsulphate
SNM – sensitive to nitrogen mustard
ss – Single strand
TAE – Tris, acetic acid, EDTA
TBE – Tris, boric acid, EDTA
TEV – Tobacco etch virus
TLS – Translesional synthesis
V(D)J – Variable Diversity Joining
YPD – yeast extract peptone dextrose

CHAPTER ONE

INTRODUCTION

1.0. INTRODUCTION

DNA interstrand crosslinks (ICL) are generated by covalently joining opposing DNA strands, and therefore represent a lethal form of DNA damage as this blocks replication and transcription of the DNA. Interstrand crosslink removal is unique in that both strands contain the damage and must be removed without the loss of genetic information. To accomplish this, cells recruit members from several DNA repair pathways to repair the damage. An overview of each of the repair pathway exploited in interstrand crosslinking repair will be discussed in the following sections. Additionally, interstrand crosslinking repair also requires DNA repair proteins that are not known to function in any other repair pathway. One such protein is Pso2. There are three human homologues of Pso2, SNM1A, SNM1B (Apollo), and SNM1C (Artemis), which will also be discussed in the succeeding sections.

1.1. DNA DAMAGE OVERVIEW

Deoxyribose nucleic acid (DNA) encodes information for directly or indirectly synthesizing all cellular components. Preservation of sequence is therefore essential for sustaining cellular functions and thus it is not surprising that cells maintain specialized mechanisms to repair any damage to DNA. DNA damage occurs in many forms including modification to nucleotide bases by processes such as oxidation, alkylation and hydrolysis; incorporation of an incorrect base during DNA replication; or esterification of the phosphodiester bond leading to single strand or double strand breaks. These damaging events

can arise from endogenous sources including DNA replication or reactive oxygen species generated intracellularly during normal metabolism and exogenous sources such as ultraviolet and ionizing radiation or chemical exposure. A typical cell undergoes hundreds of thousands of damaging events each day (Saul & Ames, 1986). In spite of this, genetic integrity is retained due to highly sophisticated cellular DNA repair mechanisms. As one might expect, the type of damage that occurs largely determines the repair pathway utilized by the cell; nonetheless, some features of repair are common including recognition of the damage, followed by signaling for chromatin remodeling, cell cycle arrest and ultimately DNA repair. If the damage cannot be repaired the cell may undergo apoptosis or be held in a senescent state. This process is vital for higher organisms given that incorrect repair of the DNA can lead to deleterious effects such as mutation or chromosomal translocation leading to tumorigenesis.

Typically each repair pathway consists of a number of members required for that particular type of DNA damage. However, DNA Interstrand crosslinking repair is unique in that it recruits proteins from other DNA repair pathways such as double strand break repair, nucleotide excision repair and translesion synthesis.

1.1.1 DNA Double Strand Break Repair

DNA double strand breaks (DSBs) occur when the phosphodiester bond is cleaved on both DNA strands and is considered the most lethal form of DNA damage. An estimated 10 double strand breaks occur in each human cell every

day (Lieber et al, 2003, Lieber & Karanjawala, 2004, Martin et al, 1985). Double strand breaks are generated primarily via replication past a single strand nick or collapsed replication forks during cell division (Kuzminov, 1995, Kuzminov, 2001). They are also, however, produced by reactive oxygen species generated during metabolism or exposure to ionizing radiation generating nicks on opposing DNA strands in close proximity (Chance et al, 1979, Richter et al, 1988, Ward, 1988). Furthermore, cellular nucleases generate DNA double strand breaks; examples include topoisomerase, RAG1/RAG2, and Spo11 (Adachi et al, 2003, Mahowald et al, 2008, Zickler & Kleckner, 1999). Regardless of how the DSBs are generated they must be repaired accurately and efficiently. Failure to do so may result in cell death or genetic translocation, leading to altered cellular function (Wang et al, 2008, Yan et al, 2007). Cells possess two main pathways for repair of double strand breaks; homologous recombination repair and non-homologous end joining (Kass & Jasin, 2010, Lieber et al, 2003, Moynahan & Jasin, 2010).

Homologous recombination requires a homologous template and therefore is primarily restricted to the S and G2 phase of the cell cycle in higher eukaryotes. After a double strand break is generated, 5' end resection is initiated by the exonuclease activity of the protein complex consisting of Mre11, Rad50, Xrs2 (NBS1 in mammalian cells) in co-operation with Sae2 (Mimitou & Symington, 2008, Zhu et al, 2008). This complex is often referred to as the MRX(N) complex. Continuation of resection is carried out by helicase Sgs2 and

the 5' – 3' exonuclease Exo1 (Mimitou & Symington, 2008, Zhu et al, 2008). The resulting 3' single strand DNA tail is coated by the single stranded binding protein replication protein A (RPA), which not only protects the ssDNA but also prevents any secondary structure formation that could act as a barrier to further repair (Sugiyama & Kowalczykowski, 2002). A filament of Rad51 on the 3' single strand tail is required for strand invasion (Sung, 1994). However, the presence of RPA prevents Rad51 loading and therefore requires the mediator protein Rad52, which displaces RPA by Rad51 (Beernink & Morrical, 1999, Sung, 1997). Homologous sequence of the intact chromosome is located by Rad51 mediated strand invasion in which the Rad51 coated ssDNA displaces one of the dsDNA strands resulting in a heteroduplex or displacement loop (D-loop). DNA polymerase then extends the invading strand from the 3' end permitting either of two potential outcomes to occur (Heller & Mariani, 2006, McIlwraith et al, 2005). In one outcome the newly synthesized single strand sequence anneals with the 3' tail at the opposing end of the break in a mechanism referred to as synthesis dependent strand annealing (Figure 1.1). In this outcome, polymerization and ligation of the DNA is then required for complete repair. Alternatively, the 3' tail of the opposing end of the break may anneal with the unpaired region of the D-loop. This 3' end is also extended by DNA polymerase resulting in formation of a Holliday junction, which is subsequently resolved to generate fully repaired chromosomes (Sung & Klein, 2006, Wu & Hickson, 2006).

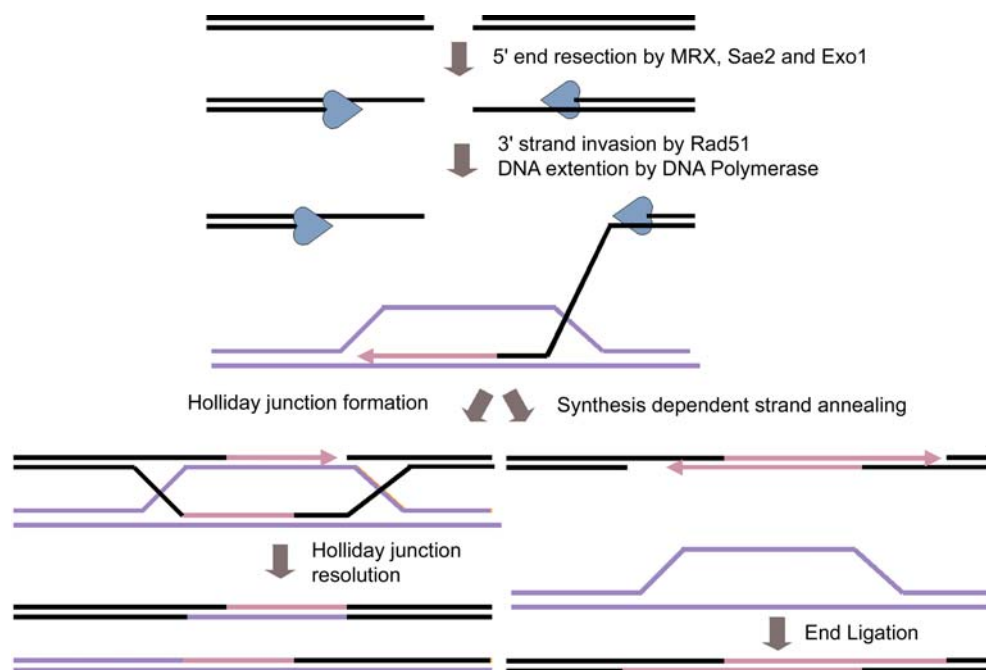


Figure 1.1 Schematic of homologous recombination. After a DSB is generated the 5' ends are resected, the 3' tail invades the homologous chromosome and DNA polymerase extends the sequence (Heller & Marians, 2006, McIlwraith et al, 2005, Mimitou & Symington, 2008, Sung, 1994, Zhu et al, 2008). Synthesis dependant strand annealing involves the newly synthesized strand annealing with the complementary sequence of the 3' tail on the opposing side of the DSB. Alternatively this 3' tail may base pair with the free strand on the homologous chromosome. DNA synthesis in this direction generates a Holliday junction, which is resolved to generate fully repaired DNA (Sung & Klein, 2006, Wu & Hickson, 2006).

Given that homologous recombination requires a homologous template for repair, this pathway is restricted from occurring during G1 and G0 when a homologous chromosome is not present. Non-homologous end joining is not restricted to any phase of the cell cycle as it is not dependent on a homologous template. Non-homologous end joining begins with binding of the heterodimer Ku70/Ku80 to double strand break ends where it recruits DNA-PKcs. Together this holoenzyme complex (DNA-PK) is thought to synapse DNA ends (Blier et al, 1993, DeFazio et al, 2002, Mimori & Hardin, 1986). DNA-PKcs is in turn responsible for the recruitment and modification of several proteins including autophosphorylation and phosphorylation of SNM1C (Artemis) and XRCC4 like factor (XLF) (Weterings & Chen, 2007). Because DNA breaks generated *in vivo* are rarely blunt ends or chemically compatible for direct ligation, additional processing enzymes are typically recruited to the site of DNA damage. Nucleotides can be added by Polymerase μ and λ while polynucleotide kinase/phosphatase (PNKP) can add or remove phosphate groups from 5'- and 3'- ends as required (Chappell et al, 2002, Gilfillan et al, 1993, Komori et al, 1993). Ultimately processing enzymes yield DNA ends that are compatible ends for ligation by Ligase IV. Although Ligase IV carried out the catalysis of strand joining, it requires interaction with XRCC4 and XLF for function (Ahnesorg et al, 2006, Grawunder et al, 1998, Wilson et al, 1997).

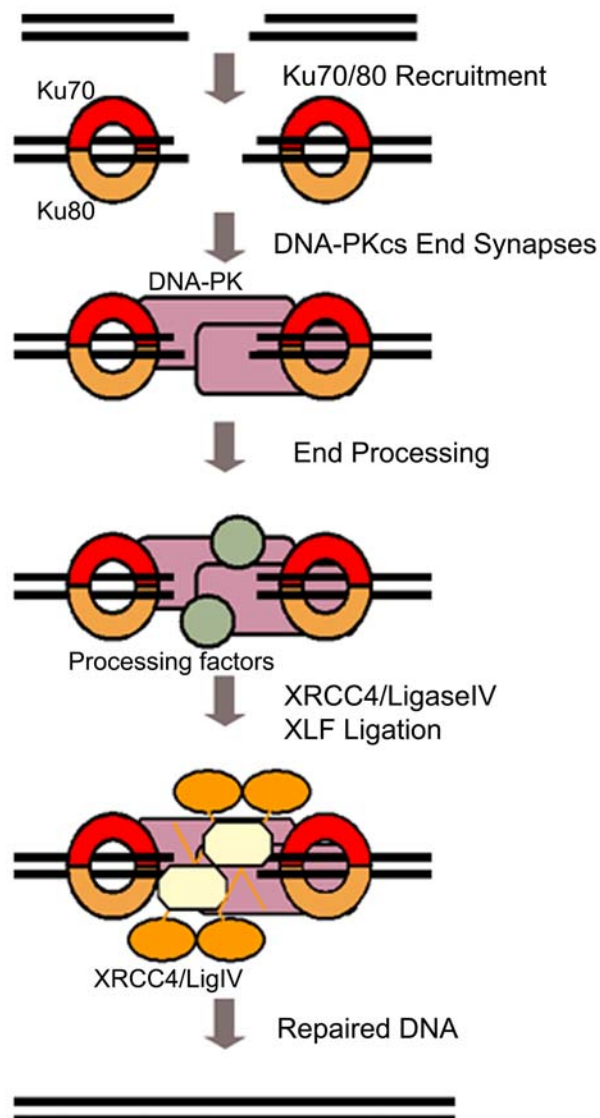


Figure 1.2 Schematic of non-homologous end joining model. Double strand break ends are recognized by the heterodimer Ku70/Ku80 (red and orange rings) that recruits DNA-PK (pink rectangle) (Blier et al, 1993, DeFazio et al, 2002, Mimori & Hardin, 1986). Various enzymes process the DNA ends (green circle) prior to ligation by Xrcc4/Ligase IV and XLF (orange and yellow complex).

1.1.2 Nucleotide Excision Repair

Exposure to ultraviolet B (UV-B) radiation can result in DNA intrastrand crosslinking between adjacent pyrimidine bases generating pyrimidine dimers. Since, damage only occurs on one strand the opposing strand is available as a template for repair. In global genome nucleotide excision repair, damage is first recognized by Rad4/Rad23 (XPC/HR23B in humans) via Rad4 binding to non-hydrogen bonded DNA bases (Clement et al, 2010); (Camenisch et al, 2009, Sugasawa et al, 1998, Trego & Turchi, 2006). This interaction further perturbs the DNA structure allowing entry of the ATP dependent 5' helicase Rad3 (XPD) and the 3' helicase Rad25 (XPB) (Guzder et al, 1994, Sung et al, 1993, Drapkin et al, 1994, Schaeffer et al, 1994, Sung et al, 1987). In transcription coupled nucleotide excision repair the damage is encountered by RNA Polymerase leading to polymerase stalling and recruitment of the nucleotide excision repair factors (Bohr et al, 1985). The damaged strand is cleaved 3' and 5' to the lesion by the single strand endonucleases Rad2 (XPG) and Rad1/Rad10 (XPF/ERCC1), respectively, while single stranded DNA on the opposing strand is protected by RPA (Bardwell et al, 1994, Habraken et al, 1995, O'Donovan et al, 1994, Staresincic et al, 2009). In human cell lines, XPF/ERCC1 has been shown to cleave approximately twenty nucleotides from the lesion while XPG cleaves only six nucleotides away (Leibeling et al, 2006). Subsequent to removal of the damaged strand, Replication Factor C (RFC) loads Proliferating Cell Nuclease Antigen (PCNA), which in turn recruits replication factors such as DNA

polymerase δ or ϵ to fill in the 24 – 32 nucleotide gap (Nichols & Sancar, 1992, Popanda & Thielmann, 1992, Shivji et al, 1992, Smith et al, 2010). The remaining nick is then sealed by DNA ligase I or DNA ligase III (Moser et al, 2007, Nocentini, 1999).

1.1.3 *Translesional Synthesis*

DNA bases can be damaged by numerous mechanisms including oxidation, alkylation, hydrolysis, and crosslinking. Unable to hydrogen bond with their complementary base, replicative DNA polymerases α , δ and ϵ generally stall when attempting to insert a complementary nucleotide. If replication stalls PCNA is ubiquitinated, leading to recruitment of various translesional polymerases, which can incorporate a nucleotide at this position and permit replication to be re-established (Lehmann et al, 2007, Masutani et al, 2000, McCulloch et al, 2004, Ogi et al, 2002, Prakash et al, 2005, Tissier et al, 2000). What makes translesional synthesis repair (TLS) unique from other DNA repair pathways is the lack of specific damage recognition of the damage.

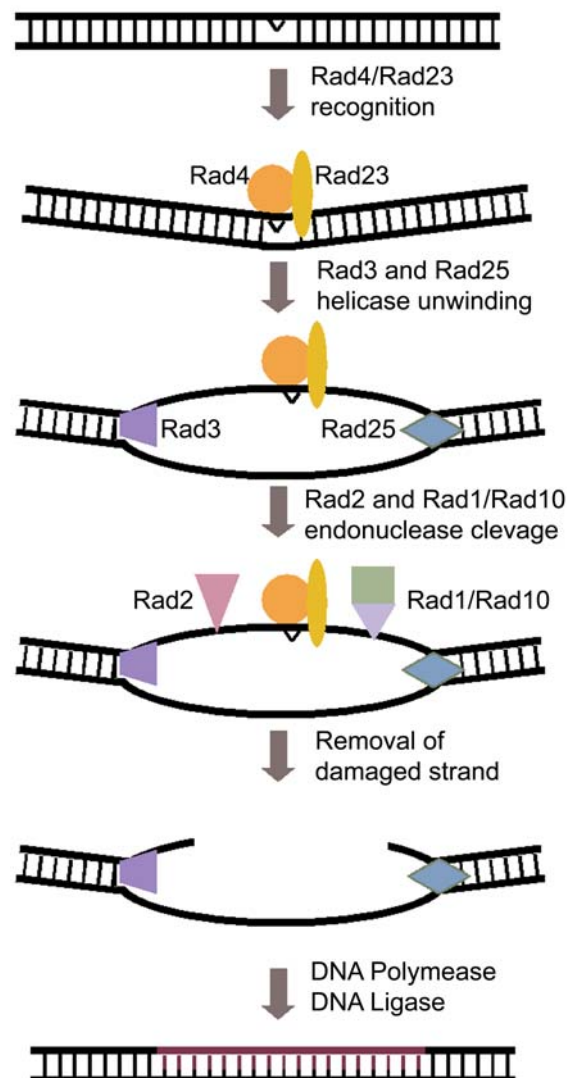


Figure 1.3 Schematic of nucleotide excision repair model. Damage is recognized by Rad4/Rad23 (Orange and yellow complex) causing DNA distortion and access of the helicases Rad3 and Rad25 (purple trapazoid and blue diamond) (Clement et al, 2010, Sugasawa et al, 1998, Guzder et al, 1994, Sung et al, 1993, Drapkin et al, 1994, Schaeffer et al, 1994, Sung et al, 1987). The single strand endonucleases Rad2 (pink triangle) and Rad1/Rad10 (purple and green complex) cleave either sides of the lesion completely removing the damaged strand (Bardwell et al, 1994, Habraken et al, 1995, O'Donovan et al, 1994, Staresincic et al, 2009). DNA polymerase fills in the gap and the nick is sealed by DNA ligases (Moser et al, 2007, Nocentini, 1999, Nichols & Sancar, 1992, Popanda & Thielmann, 1992, Shivji et al, 1992).

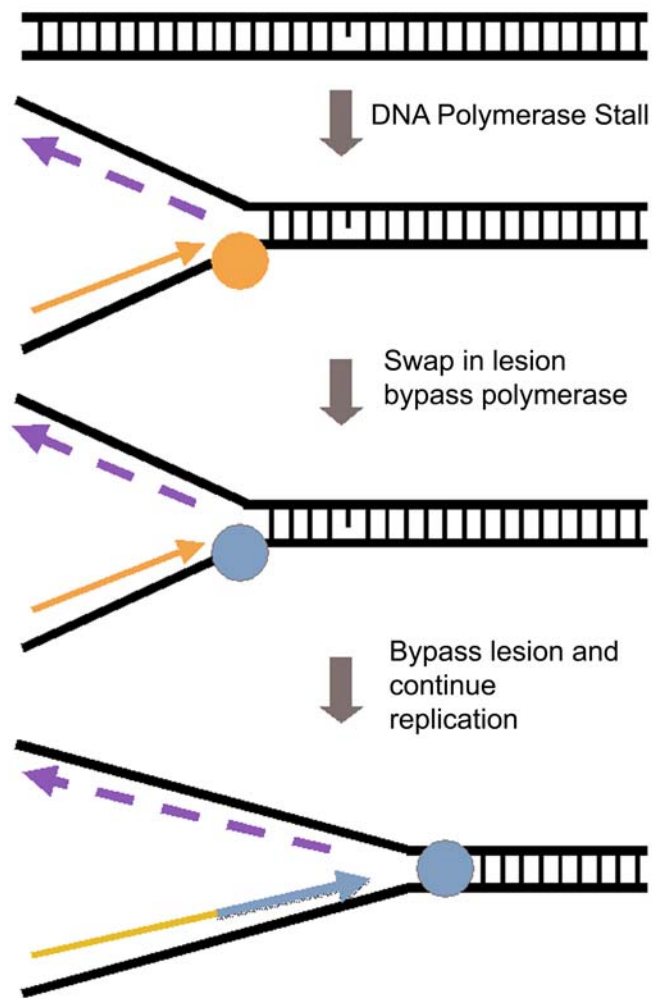


Figure 1.4 Schematic of translesional synthesis. DNA polymerase (orange circle) stalls and a translesional polymerase (blue circle) is swapped in which can bypass the damage. Once the damaged base is passed, leading and lagging strand polymerases continue replication.

1.1.4. *Repair DNA hairpin structures*

Many types of DNA structures are generated in response to DNA damage, repair and recombination that require processing via specialized nucleases. One such class of structures are DNA hairpins formed during V(D)J recombination, palindrome extrusion, DNA transposition, and some types of double strand breaks.

DNA hairpins pose a significant challenge to genome stability. Once formed, such structures must be removed and/or opened by specialized nucleases to permit subsequent repair. DNA hairpins are generated in a variety of ways, with perhaps the most well characterized being programmed hairpin formation resulting from V(D)J recombination (McBlane et al, 1995, Roth et al, 1992). Hairpin structures capping DNA ends are also formed at inverted repeat sequences and in some instances, as intermediates during DNA repair (Lobachev et al, 2007). Regardless of how hairpins are generated, these ends must be appropriately processed before ligation can occur. Failure to do so results in accumulation of double strand breaks, and thus a potentially lethal cellular event (Lobachev et al, 2007).

Cells maintain at least two mechanisms for removal of DNA hairpins. The first involves the Mre11/Rad50/Xrs2 in yeast (MRX) and Mre11/Rad50/Nbs in humans (MRN) complex, which is well characterized for its essential role in processing double strand breaks (DSB) during repair (Huang & Dynan, 2002, Moore & Haber, 1996). This complex has 3'-exonuclease activity necessary for

DSB repair as well as a structure-specific endonuclease hairpin opening activity important for stability of inverted repeat sequences (Lobachev et al, 2002, Paull & Gellert, 1998). In conjunction with Sae2, MRX has been shown to catalyze the entire removal of DNA hairpin structures (Figure 1.5). MRX/Sae2 functions at damaged cruciform structures that have been converted into DNA hairpins with a single strand nick distal to the hairpin cap (Lengsfeld et al, 2007). MRX is able to open a gap from the available nick using its 3'-exonuclease activity and stimulates Sae2 to cleave the resulting ssDNA on the opposing strand, thereby completely removing the hairpin structure. Artemis (SNM1C) represents a second specialized nuclease capable of processing DNA hairpins; however, its function appears to be largely restricted to repair associated with the non-homologous end-joining (NHEJ) DSB repair pathway following V(D)J recombination. The structure-specific endonuclease activity of Artemis (SNM1C) generates nicks at or very close to the apex of DNA hairpin structures, generating free ends for further processing and repair (Ma et al, 2002). A similar activity has also been reported for the MRX/Sae2 complex in yeast (Lobachev et al, 2002).

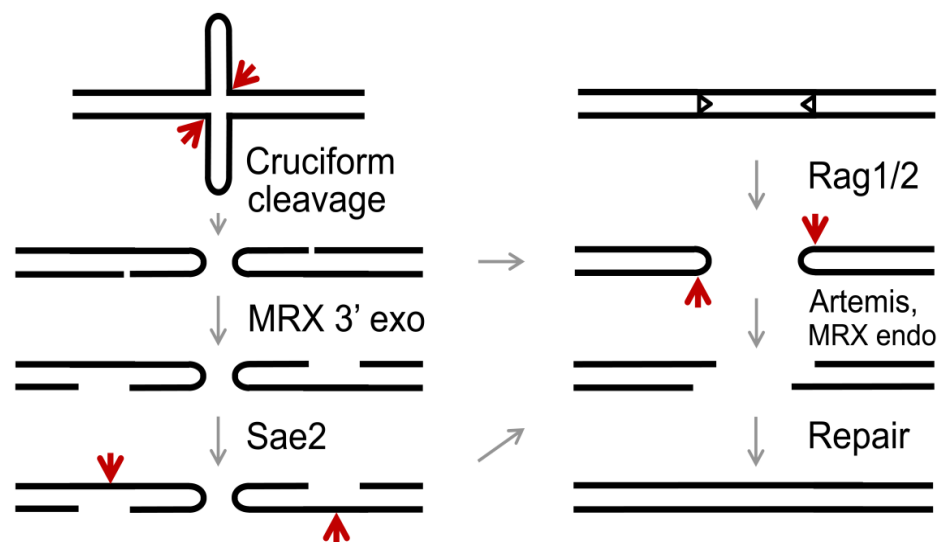


Figure 1.5 Mechanisms of DNA hairpin processing. DNA hairpins are formed both at inverted repeat sequences following cruciform extrusion and cleavage (Meuth *et al*, 1989; Stark *et al*, 1989) and also during V(D)J recombination via RAG1/2-mediated coding joint cleavage (Roth *et al*, 1992; McBlane *et al*, 1995). Following cruciform resolution, the nicked hairpin structure can either be ligated directly to form a completely closed hairpin or further processed by the 3' exonuclease activity of the MRX complex (Paull and Gellert, 2000). Sae2 is then able to recognize and cleave the single strand region generated by Mre11, removing the entire hairpin cap (Lengsfeld *et al*, 2007). Closed hairpins structures can be opened by the endonuclease activity of SNM1C (Artemis) or the MRX/N complex (Ma *et al*, 2002; Paull and Gellert, 2000).

1.1.5. DNA interstrand crosslinking Repair

Interstrand crosslinks (ICLs) are a form of DNA damage that arise as a consequence of bifunctional alkylating agents covalently binding to opposing DNA strands, thereby joining them together. The resulting interstrand crosslinks prevents the replication and transcription of DNA, making ICLs lethal to the cell (Scharer, 2005). A number of different chemicals are able to form an ICL including: nitrogen mustard, psoralen, mitomycin C, carmustin, cisplatin, and malondialdehyde (reviewed in Lawley & Phillips, 1996). Nitrogen mustards directly react with guanine residues at GNC sequences on opposing strands and result in only minor distortion of the DNA duplex (Guainazzi et al, 2010). Psoralen agents first intercalate in DNA and only form crosslinks upon UV activation at AT sequences again causing only minor DNA distortion (Guainazzi et al, 2010, Rink & Hopkins, 1995). Mitomycin C reacts with guanine residues from opposing strands in the minor groove, at GC sequences, slightly widening this groove (Hwang et al, 1996, Sinden & Hagerman, 1984, Spielmann et al, 1995, Stern, 2007). Cisplatin also reacts with guanine in GC sequences, however; in this case major distortion to the DNA is created (Tomasz et al, 1986, Tomasz et al, 1987). Carmustine generates crosslinking across a G-C base pair with very little DNA perturbation (Coste et al, 1999a, Huang et al, 1995, Jamieson & Lippard, 1999). Since carmustine can cross the blood brain barrier it is often used in the treatment of brain tumors (Ludlum, 1997). Finally,

malondialdehyde reacts with guanine at either GC in the minor groove or CG in the major groove of opposing strands.

In order to preserve the integrity of genomic information and prevent cell death, a specialized repair pathway exists for repair of ICLs. While the mechanism of ICL repair is not fully understood it is believed to begin with recognition of the damaged site followed by incision of the DNA, in turn creating a double strand break (Wood, 2010). It has been proposed that the DSB is generated as a result of endonucleases nicking the DNA flanking the lesion followed by replication past this nick. In support of this, double strand breaks arise less frequently if ICL repair endonucleases are lacking and more frequently in actively replicating cells (Johannessen et al, 2008).

Several proteins are known to be involved in repair of ICLs, most of which are also involved in other DNA repair pathways such as nucleotide excision repair, homologous recombination, and translesional synthesis while others are unique to the ICL repair pathway (Magana-Schwencke et al, 1982, McHugh et al, 2000). The generally accepted model of ICL repair, illustrated in Figure 1.6, is initiated by recognition of the lesion by the nucleotide excision repair protein Rad4/Rad23 (XPC/RAD23B in humans). Subsequently, Rad2 nicks one of the DNA strands 3' to the ICL and Rad1/Rad10 cleaves the same strand 5' to the damage. The Rad1/Rad10 complex is, however, believed to play a more critical role than RAD2 and some groups suggest that Mus81 may, in fact, cleave the DNA 3' to the ICL (Hinz, 2010b, Ho & Scharer, 2010, Wood, 2010). The nicked

strand, containing the damage, is flipped out and the opposite strand serves as a template for various translesional polymerases such as Pol ζ , Pol η , or Pol κ which are able to read through the ICL damaged nucleotide (McPherson et al, 2004). Similar nicking reactions then occur on the opposite strand, completely removing the sequence containing the ICL. Polymerases fill in this gap while ligases seal the nick. During cellular replication, DNA Polymerase may encounter these nicks during repair and generate a double strand break (Albertella et al, 2005, Chen et al, 2006, Minko et al, 2008, Nojima et al, 2005, Sarkar et al, 2006, Shen et al, 2006, Sonoda et al, 2003, Wu & Hickson, 2006, Yamanaka et al, 2010). This broken end invades the newly repaired DNA utilizing homologous recombination machinery, restarting the replication fork (Hinz, 2010a, Niedernhofer et al, 2004). During replication-independent ICL repair, double strand breaks are not generated and homologous recombination is not required (Hinz, 2010a).

In addition to proteins from HHR, NER, and TLS pathways, ICL repair also requires unique proteins which are thought to function solely in this repair pathway. One such protein is Pso2, a nuclease belonging to the β -CASP family of enzymes.

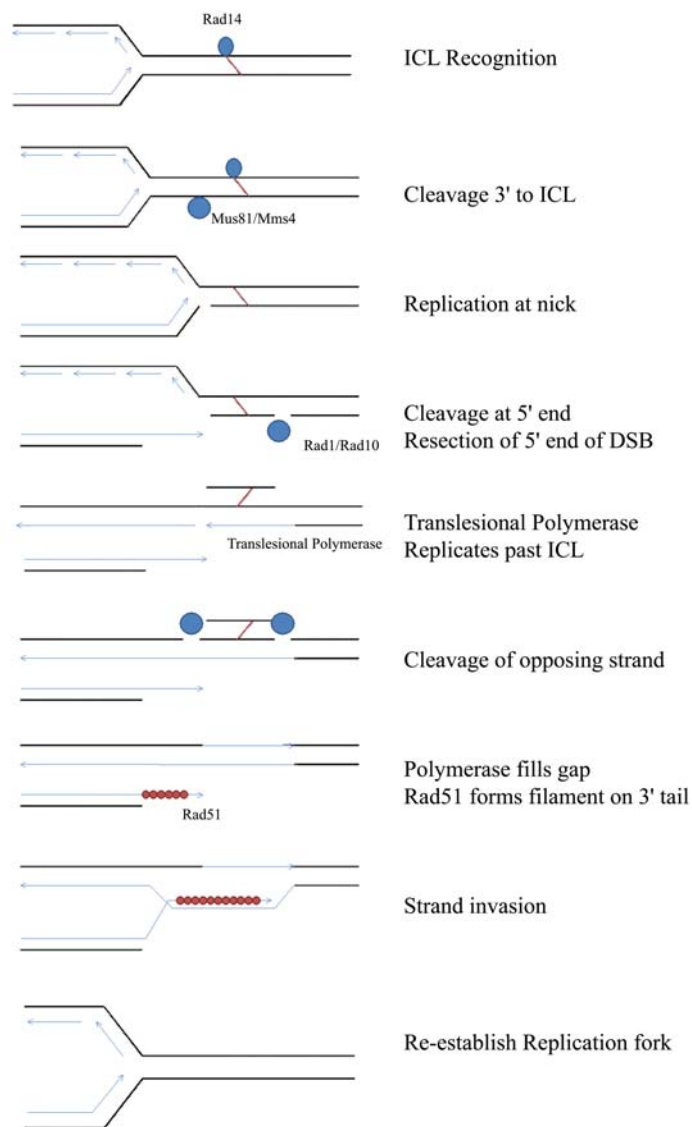


Figure 1.6 Schematic of proposed model of eukaryotic ICL repair. Replication stalls as damage is recognized by Rad14. Cleavage occurs on both sides of the ICL lesion on one strand, the damaged region is flipped out, and the opposing strand serves as a template for translesional polymerase machinery that can bypass the ICL. Next cleavage occurs on the opposite strand to completely remove the ICL damage. DNA polymerase fills in this gap. As replication encounters the nick a DSB is generated which can invade the newly repaired strand restarting the replication fork.

1.2 β -CASP FAMILY

All members of the β -CASP family are associated with nucleic acid processing and repair. Members of this family contain a β -CASP domain. The β -CASP domain belongs to a specialized subset of the metallo- β -lactamase superfamily, which is involved in a wide variety of processes ranging from cleavage of lactam, rings in antibiotic resistance to detoxifying byproducts of carbohydrate and lipid metabolism (McHugh et al, 2000, Sarkar et al, 2006). The metallo- β -lactamase fold consists of a central core consisting of a β -sandwich (strands 1-7 and 8-12) flanked on either side by five α helices. A metal binding site is located on one side of the β -sandwich and is coordinated by residues from five highly conserved motifs including an aspartic acid at the end of β -stand 5, an HxHxDH motif, and five additional motifs each of which contain a single conserved residue succeeding a β -strand (H, D, D/E, H, V/H) (Daiyasu et al, 2001). These structural elements are presumed to be involved in both enzymatic activity and domain stability. The distinguishing feature of the β -CASP domain is a large insertion, often referred to as the CASP insertion, replacing the fifth motif of the metallo- β -lactamase domain. This CASP insertion has three conserved motifs of its own consisting of an acidic residue, a histidine, and either a histidine or valine each following a β -strand (Callebaut et al, 2002). This last residue determines specificity towards RNA and DNA respectively. The presence of the CASP insertion is thought to be responsible for imparting unique properties to β -CASP domains. The crystal structure of mRNA processing enzyme, CPSF-73,

revealed that the CASP domain is comprised of a 7-stranded parallel β -sheet surrounded by three α -helices forming a lid over the metallo- β -lactamase active site (Mandel *et al*, 2006) (Figure 1.7). To date there have been no structures determined for DNA processing β -CASP enzymes.

Of the DNA processing β -CASP members there is a subset referred to as the SNM1 family consisting of yeast Pso2 (Snm1) as well as the three human homologues SNM1A, SNM1B (Apollo), and SNM1C (Artemis) (Callebaut *et al*, 2002).

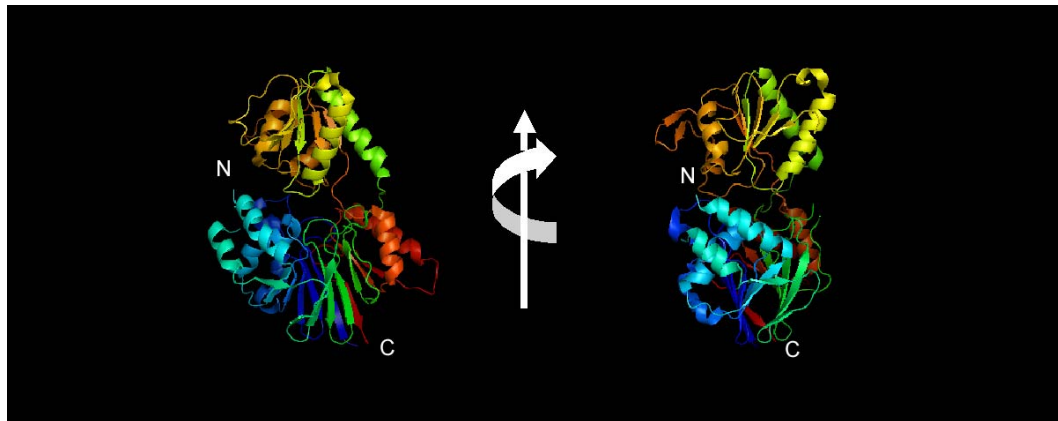


Figure 1.7 Crystal Structure of CPSF-73. The structure of CPSF-73 (PDB accession code: 2I7T) reveals a β -sheet flanked by alpha helices forming a lid at the top of the metallo- β -lactamase domain.

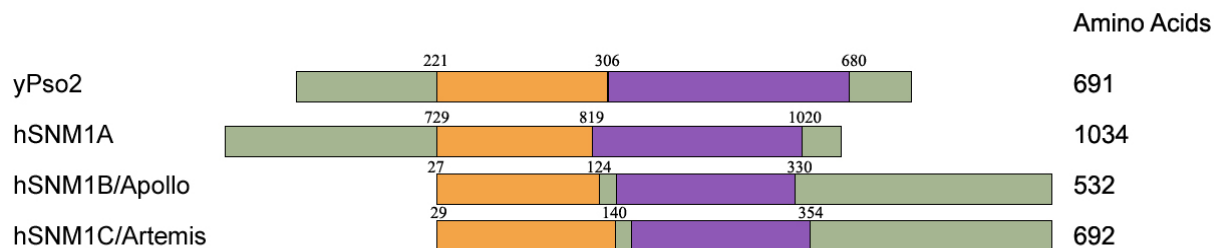


Figure 1.8 Alignment of the SNM1 family of proteins. The conserved metallo- β -lactamase domain is shown in orange and the CASP insertion shown in purple.

1.2.1 *Pso2*

Despite having an important role in ICL repair, very little is currently known about how SNM1A (*Pso2*) functions inside the cell. *PSO2* (*SNM1*) was first identified in a screen of yeast mutations that greatly increased sensitivity to the crosslinking agent, psoralen (Henriques *et al*, 1980). *SNM1* was later found to be allelic to *PSO2*, which when deleted rendered cells sensitive to nitrogen mustard (Cassier-Chauvat *et al*, 1988 and Ruhland *et al*, 1981). Since then *pso2* null strains have demonstrated sensitivity to a range of interstrand crosslinking agents including chlorambucil, cyclophosphamide, cisplatin, oxaliplatin and mitomycin C (Henriques and Moustacchi, 1981; Ruhland *et al.*, 1981). The β -CASP domain, which is localized to the C-terminus, is essential for ICL repair. Amino acid substitutions of conserved residues within the β -CASP domain result in increased ICL sensitivity and decreased activity (Cattell *et al*, 2010). A ‘free’ N-terminus is also required for repair, as various N-terminal protein fusions have been shown to decrease survival following exposure to ICL agents. The N-terminus is poorly conserved and contains a putative zinc finger domain, which does not appear to be present in the other human homologues. *Pso2* was originally assigned to the nucleotide excision repair epistasis group, yet the paradox remains that while NER enzymes are required for incision events, mutations in *pso2* result in an accumulation of DNA double strand breaks (DSBs) following exposure to ICL inducing agents suggesting that *Pso2* acts downstream of the incision event in ICL repair (Hejna *et al*, 2007, Li & Moses, 2003, Li *et al*,

2005). This raises the possibility that Pso2 acts upon intermediates generated by NER prior to successful repair. Conversely, cells harboring a *pso2* deletion exhibit no significant sensitivity to other DNA damaging agents including ionizing radiation, UVC exposure, and HO endonuclease, suggesting a specialized role for Pso2 in the repair of double strand breaks formed during repair of ICL lesions (Henriques & Moustacchi, 1981, Li & Moses, 2003). The specific requirement of Pso2 in ICL repair suggests that these breaks may be in some way different from other forms of DNA breaks. In fact, PSO2 is only transcribed upon addition of ICL inducing agents and not other forms of DNA damage. Under non-stressed conditions, Pso2 transcripts are very low, at less than 1 copy per cell; however, following ICL damage transcription is increased four-fold. This is not observed upon addition of other DNA damaging agents. Pso2 transcription is regulated by DUN1, a kinase that is responsible for transcriptional regulation of various cell cycle checkpoint genes.

Yeast Pso2 has three vertebrate paralogues, which make up the SNM1 family. While each protein contains a conserved β -CASP domain, the location of this domain can be at the N- or C-terminus of the protein. Every SNM1 enzyme also contains one additional domain, which is unique to each member and not conserved amongst the group. The human SNM1 enzymes all appear to function as nucleotide processing enzymes. In addition, they each possess additional roles such as telomere maintenance and cell cycle checkpoint described in the following sections (Barber et al, 2005, Henriques & Moustacchi,

1980, Lam et al, 2008, Li & Moses, 2003, Akhter et al, 2004, Liu et al, 2009, van Overbeek & de Lange, 2006, Freibaum & Counter, 2006, Lenain et al, 2006b, van Overbeek & de Lange, 2006).

1.2.2 *SNM1A*

SNM1A is the largest member of the SNM1 family at 116 kDa (Geng et al, 2007). It is the most closely related to Pso2 retaining the highest sequence similarity at 39% and appears to have a similar functional role in ICL repair (Akhter et al, 2004). Furthermore human SNM1A can complement a yeast *pso2* null strain in ICL resistance (Dronkert et al, 2000b, Ishiai et al, 2004a). SNM1A depletion leads to ICL sensitivity in three mammalian systems analyzed; mouse, chicken, and human. Under non-stressed conditions knock out mice are viable and appear phenotypically normal. Fibroblasts isolated from SNM1A depleted mice have been shown in three independent studies to be sensitive towards mitomycin C (MMC) but not towards other ICL agents (Hazrati et al, 2008a). This suggests, that as in yeast, redundant pathways for ICL repair are present. Although, chicken cell lines disrupted for SNM1A display sensitivity towards a broader spectrum of ICL agents, this sensitivity is increased when SNM1A is depleted in combination with other proteins including SNM1B (Apollo), XRCC3, Rad18, and FANCC; further suggesting redundant repair pathways (Akhter et al, 2004, Dronkert et al, 2000a, Hemphill et al, 2008). Finally, MMC sensitivity is also observed in human fibroblasts treated with RNAi towards SNM1A.

These studies clearly place SNM1A as an important player in repair of ICL damage. Analysis of SNM1A has demonstrated that a long 5'- untranslated region (UTR) controls translation and allows upregulation to occur during mitosis (Ishiai et al, 2004a). Nuclear foci appear following DNA damage, co-localizing with proteins known to be involved in recognition of double strand breaks including Mre11 and 53BP1 (Zhang et al, 2002). In fact, SNM1A has a proposed role in regulation cell cycle checkpoint as fibroblasts fail to arrest at prophase when SNM1A is depleted (Richie et al, 2002). This may account for chromosomal instability observed in SNM1A deficient cells (Akhter et al, 2004).

Biochemical studies have revealed that SNM1A, like yeast Pso2, displays 5' to 3' exonuclease activity (Hemphill et al, 2008). As with Pso2, mutations to conserved residues of SNM1A within the β -CASP abolish nuclease activity. Preference towards ss-DNA is observed in SNM1A, which is not present in Pso2 and the other human SNM enzymes (Hejna et al, 2007). Importantly, SNM1A was not shown to possess any endonuclease function (Hejna et al, 2007).

1.2.3 SNM1B (Apollo)

Human SNM1B (Apollo) shares 33% similarity towards yeast Pso2 and also plays a functional role in ICL repair (Hejna et al, 2007). However, unlike Pso2 and SNM1A; SNM1B (Apollo) is thought to function prior to incision (Demuth et al, 2004). Similar to SNM1A, all cell lines tested display sensitivity towards MMC, however, there are conflicting reports within the literature regarding sensitivity towards ionizing radiation (Bae et al, 2008, Liu et al, 2009).

A role for SNM1B (Apollo) in cell cycle checkpoint has also been demonstrated for SNM1B (Apollo) at both G2 and prophase (Bae et al, 2008, Demuth et al, 2004, Ishiai et al, 2004b, Liu et al, 2009). In fact, phosphorylation defects are observed in Chk1 in the absence of SNM1B (Apollo) further supporting a role for SNM1B (Apollo) in regulation of cell cycle checkpoint (Liu et al, 2009, van Overbeek & de Lange, 2006).

SNM1B (Apollo) has been shown to interact with the Shelterin complex, specifically TRF2, and is therefore proposed to be involved in telomere maintenance (Anders et al, 2009). Ubiquitination and degradation of SNM1B (Apollo) occurs in the absence of TRF2, suggesting that TRF2 is important for SNM1B (Apollo) stability (Freibaum & Counter, 2006, Lenain et al, 2006b, van Overbeek & de Lange, 2006). Given that SNM1B (Apollo) abundance at telomeres is far less than that of TRF2 it would appear that SNM1B (Apollo) may act as an accessory protein (Freibaum & Counter, 2006). Upon DNA damage, both SNM1B (Apollo) and TRF2 localize to damage sites (van Overbeek & de Lange, 2006). When SNM1B (Apollo) is depleted by RNAi, DNA damage response factor accumulates at telomere ends during S phase and when SNM1B (Apollo) is depleted in combination with TRF2 chromosome fusions and growth defects occur (Demuth et al, 2008). Given that SNM1B (Apollo) appears to function during S phase, it has been proposed that SNM1B (Apollo) may process post replication chromosomes so they are distinct from broken DNA ends.

Biochemical studies have shown that like SNM1A, SNM1B (Apollo) is also a 5' exonuclease but with little substrate preference (van Overbeek & de Lange, 2006).

1.2.4. SNM1C (Artemis)

SNM1C (Artemis) is the best characterized of the SNM1 proteins and displays highest similarity to SNM1B (Apollo). Additionally, both SNM1B (Apollo) and Artemis have related domain organization with their β -CASP domains located at their N-terminus (Lenain et al, 2006b). Unlike SNM1A and SNM1B (Apollo), Artemis is not involved in ICL repair but rather plays a role in non-homologous end joining in both DSB repair as well as V(D)J recombination. Patients lacking functional Artemis develop radiosensitive severe combined immunodeficiency (RS-SCID) and lack mature T and B lymphocytes as well as increased sensitivity towards radiation (Moshous et al, 2001). V(D)J recombination is a specialized pathway in which T cells and B cells recombine gene segments coding for variable regions of T cell receptors and B cell antibodies, respectively. RAG1/RAG2 generated double strand breaks containing capped DNA hairpins opened by Artemis following phosphorylation by DNA-PKcs. This DNA hairpin opening is absolutely required for further repair by non-homologous end joining (Moshous et al, 2001, Rooney et al, 2002a). *In vitro*, Artemis has been shown to open DNA hairpins 2 nucleotides from the nick on the 3' side of the hairpin (Ma et al, 2002). Given that Artemis is also able to act on various DNA structures such as DNA overhangs, flaps, loops, gaps,

hairpins, and oxidative DNA lesions, it is believed that Artemis processes incompatible ends generated by damaging agents (Goodarzi et al, 2006, Jeggo & O'Neill, 2002, Ma et al, 2002). This may account for the fact that Artemis is only required for repair of a subset of DSBs generated by ionizing radiation (Ma et al, 2002, Poinsignon et al, 2004). Artemis contains a C-terminal domain that does not appear to be present in any other protein. This domain is highly phosphorylated by DNA-PKcs at multiple SQ/TQ sites, activating Artemis endonuclease activity (Darroudi et al, 2007, Riballo et al, 2004, Wakasugi & Sancar, 1999, Wang et al, 2005). It is believed that this post-translational modification alleviates suppression of endonuclease activity. In support of this suggestion, complete removal of the C-terminal domain results in constitutive activation of the endonuclease activity of Artemis (Ma et al, 2005). Interestingly, the C-terminal domain of Artemis is only required for V(D)J recombination but not for DSB repair (Niewolik et al, 2006). Analogous to the other human SNM1 proteins, Artemis also appears to play a role in cell cycle checkpoint and perhaps this C-terminal domain is required for this function as well. In particular, Artemis is important in the regulation of Cyclin B-Cdk1 necessary for G2/M checkpoint (Poinsignon et al, 2004).

Artemis displays 5' exonuclease activity which surprisingly is not dependent on the conserved β -CASP residues shown to be critical for endonuclease function including repair of hairpin ends formed during V(D)J recombination (Geng et al, 2007).

1.3. THESIS OBJECTIVES

Defining how DNA repair pathways work is central to the understanding development of certain diseases including tumor formation. This knowledge is also vital for developing strategies to treat such diseases. ICL agents have been used clinically to combat tumourgenesis, given the lethality in actively replicating cells. However, given the high mutation rate in cancer cells, upregulation of repair proteins has lead to resistance of these drugs and therefore understanding their roles in DNA repair is useful for future drug development.

ICL agents pose a serious threat to cell survival and all organisms possess repair pathways to deal with this damage. Although the key proteins in ICL repair have been identified, the eukaryotic mechanism for how these lesions are removed is not fully understood. Proteins from various repair pathways are recruited to collectively remove the ICL lesion. Interestingly, a small group of proteins, not assigned to any additional repair mechanism are required for ICL repair. One such protein is Pso2. In order to understand Pso2 function in removal of ICLs, *S. cerevisiae* was chosen as a model eukaryotic organism. This system is simple and at the same time retains similarity to higher eukaryotes.

Pso2 is required for ICL repair yet its function in this repair pathway is unclear. It has been proposed that Pso2 activity is required for processing the DSB generated by ICL damage or repair but not other forms of DSBs. The question then becomes what DNA intermediate is generated during ICL repair that Pso2 is uniquely suitable to act upon? To begin addressing this question,

purified Pso2 activity was analyzed towards several DNA intermediates that may be encountered during ICL repair. Interestingly, Pso2 was found to possess an efficient endonuclease activity specific to DNA hairpin structures. It was further demonstrated that this activity was required *in vivo* for restoring genomic DNA when double strand breaks are generated containing covalently closed DNA hairpins. Thus, as in the case of human SNM1C (Artemis), yeast Pso2 appears to retain two distinct nuclease activities, an exonuclease activity able to degrade DNA from a free 5' end and an endonuclease activity specific to DNA hairpin structures. These findings provide the first evidence for Pso2 functioning outside of ICL repair and suggest that Pso2 and hSNM1A may function at least in part during ICL repair by processing DNA intermediates including DNA hairpins and/or hairpin-like structures, generated directly or indirectly by ICL damage.

To identify if Pso2 endonuclease activity was required for ICL repair, the DNA hairpin opening β -CASP domain of Artemis was analyzed for its ability to restore the repair defect in a *pso2* deficient strain. Complementation studies revealed that this domain was able to suppress the ICL sensitivity in this strain, suggesting that Pso2 hairpin opening endonuclease cleavage plays an important role in ICL repair. This was further verified by the observation that DNA hairpins accumulate subsequent to ICL damage and persist in the absence of Pso2. These findings reveal a novel aspect of eukaryotic ICL repair in which DNA hairpin structures are generated as a consequence of ICL damage and/or repair and elucidates the requirement of the specialized nuclease, Pso2.

CHAPTER TWO
PSO2 IS A 5' EXONUCLEASE DEPENDANT ON
THE PRESENCE OF A 5' PHOSPHATE

2.1. Abstract

DNA interstrand crosslinks (ICLs) are lethal to cells due to the prevention of chromosome replication. A number of proteins, including those functioning in nucleotide excision repair (NER), homologous recombination repair (HRR), and translesional/ post replicative repair (TLS/PRR), are involved in the removal of ICL lesions. Pso2 belongs to a small group of proteins thought to function solely during ICL repair. In this study, results from analysis of Pso2 nuclease activity shows that Pso2 displays 5' exonuclease activity towards a variety of substrates including ssDNA, dsDNA having various ends, and nicked DNA. Exonuclease activity, but not substrate binding, is dependent on the presence of a 5' phosphate. Pso2 is also shown to be a processive and able to degrade DNA substrates to a single nucleotide. The β -CASP domain constitutes the catalytic domain of Pso2, required for nuclease activity as well as ICL resistance. These findings provide evidence for Pso2 functioning in ICL repair by processing DNA intermediates generated during repair.

2.2 Introduction

Although Pso2 was identified over 30 years ago, in a screen identifying mutations that render cells sensitive to ICL inducing agents, the function of Pso2 in ICL repair is still poorly understood (Ma et al, 2002, Pannicke et al, 2004). Pso2 has been suggested to function downstream of the ICL ‘unhooking’ event, given that cells deficient in *pso2* result in an accumulation of DNA double strand breaks (DSBs) following ICL exposure (Henriques & Moustacchi, 1980). These DSBs must possess unique characteristics requiring Pso2 processing as *pso2* deletion exhibit no significant sensitivity to other forms of DSBs including ionizing radiation, UVC exposure, and HO endonuclease (Li & Moses, 2003). The lack of a phenotypic defect in *pso2* Δ strains under non-stressed conditions or conditions that generate DSBs gave rise to the idea that Pso2 functions solely during ICL repair. Pso2 contains 5’ exonuclease activity; nonetheless, the presence of various 5’ exonuclease activities within the cell would suggest that this exonuclease activity is not the function Pso2 utilizes in repair of ICL generated damage. Pso2 contains an N-terminal zinc finger domain consisting of four cysteine residues as well as a C-terminal β -CASP domain containing several highly conserved residues important for catalytic activity.

Despite its specialized role in repairing this lethal form of DNA damage very little is known about the function of Pso2. Understanding the enzymatic and biochemical properties of Pso2 is important not only for defining its role in ICL

repair but also for understanding the mechanism by which other β -CASP domain-containing proteins function.

In order to further define the role and mechanism of Pso2 in ICL repair, I have conducted a thorough characterization of the exonuclease activity of Pso2. This analysis revealed that Pso2 is a highly processive 5'-exonuclease; able to utilize a variety of DNA substrates, providing a free 5' phosphate is available. Limited proteolysis of Pso2 revealed that the β -CASP domain adopts a stable conformation. This domain was further shown to be required but not sufficient for nuclease activity. The zinc finger domain of Pso2 was not required for DNA binding or exonuclease activity *in vitro*, and led to further increase in ICL resistance.

2.3. Materials and Methods

2.3.1. Plasmid Construction, Expression, and Purification of Pso2

The *Saccharomyces cerevisiae* Pso2 gene from pQE32 (generously provided by James Hejna, Department of Molecular and Medical Genetics, Oregon Health and Sciences University) was amplified by PCR and cloned into pDEST14 and pYES-DEST52 expression vectors using Gateway cloning technology (Invitrogen). The *Escherichia coli* cell line Rosetta (DE3) pLysS (Novagen), harboring the *PSO2* expression vector, was grown at 37°C prior to induction with 2 mM IPTG at 16°C overnight. Cells were harvested, washed with 1X PBS, and resuspended in lysis buffer (50 mM sodium phosphate pH 7.0, 500 mM NaCl, 3 mM β -mercaptoethanol, 1% Triton X-100). Preceding and

subsequent to lysis via French press, 1 mM PMSF, 1 mM benzamidine-HCl, 1 μ M pepstatin, and 0.1 μ M leupeptin were added to the cell suspension. Following centrifugation at 50 000 g for 40 min, the supernatant was injected onto a 5 mL, Ni²⁺ charged HiTrap Chelating column (Amersham) that was pre-equilibrated with equilibration buffer (50 mM sodium phosphate pH 7.0 and 500 mM NaCl). Subsequent washes with equilibration buffer supplemented with 15 mM, 30 mM, and 45 mM imidazole occurred before elution in equilibration buffer containing 150 mM imidazole. DTT and EDTA were added to the pooled fractions to a final concentration of 5 mM and 1 mM, respectively. The resulting eluent was diluted to 100 mM NaCl and injected onto a 5 mL HiTrap Q sepharose column (Amersham) equilibrated with 50 mM sodium phosphate pH 7.0, 100 mM NaCl, 1 mM EDTA, and 5 mM DTT. Pso2 was eluted in a gradient from 100-200 mM NaCl and then exchanged into buffer containing 10 mM Tris pH 7.0, 100 mM NaCl, and 5 mM DTT prior to concentrating by ultracentrifugation (30 MWCO, macrosep). Samples were frozen at -80°C in 10% (v/v) glycerol.

The point mutants *pso2* C166A/C168A, D252A, and H611A were created using site directed mutagenesis (QuikChange, Stratagene). The truncation mutants 1-188 (*pso2* Δ C) and 188-661 (*pso2* Δ N) were generated by amplifying these gene fragments with PCR primers containing flanking 5' TEV-AttB1 and 3' AttB2 or 5' Shine delgarno-AttB1 and 3' HIS₆-AttB2 sites and then further recombined into pDonor201. This vector was used to recombine into the yeast

expression vector pYES-DEST52 and *E. coli* expression vectors pDEST15 and pDEST14, respectively using Gateway technology (Invitrogen). The purification of *pso2* Δ N was performed in the same manner as wild type. The purification of *pso2* Δ C differed following immobilized metal affinity chromatography. Briefly, the eluted protein was diluted 5 fold into 1X PBS, 5 mM DTT, and 0.03% Lauryldimethylamine-oxide (LDAO), injected onto a GSTrap FF column, and eluted with 50 mM, Tris pH 8.0, 40 mM reduced glutathione, 250 mM NaCl, and 0.03% LDAO. TEV proteolysis was used to remove the N-terminal GST fusion by incubating the protein with 0.02 mg/mL TEV protease in the presence of 50 mM Tris 8.0 and 0.5 mM EDTA for 1 hour 40 minutes at room temperature. The reaction was ended by the addition of 2 mM benzamidine HCl and the protein was injected onto a HiTrap Q sepharose column equilibrated with 50 mM sodium phosphate pH 7.0, 100 mM NaCl, 1 mM EDTA, and 5 mM DTT. *pso2* Δ C eluted using a NaCl gradient at 500 mM NaCl.

Quantification of Pso2 was performed by diluting Pso2 in 6 M guanidine-HCl, and reading the absorbance at 276 nm, 278 nm, 280 nm, and 282 nm. The highest absorbance reading was at 280 nm and therefore the accurate protein concentration was determined using the method described by Pace *et al.* (1995). Purity and concentration validation was determined using Coomassie staining and Western blot analysis using mouse α His followed by goat α mouse hydrogen peroxide conjugated (HPC).

2.3.2. *Partial Proteolysis and N-Terminal Amino Acid Sequencing*

Limited digestion was carried out by incubating 96 µg of purified Pso2, containing a C-terminal His₆ fusion, with chymotrypsin in increasing amounts of 0.06, 0.12, 0.24, 0.48, 0.96, 1.92, and 3.84 ng in 80 µL reactions containing 50 mM Hepes pH 7.5, 40 mM KCl, and 10 mM MgCl₂. The reactions were incubated for 5 min at room temperature and the digestions were terminated by the addition of Laemmli buffer with 2.5 mM phenylmethanesulfonylfluoride (PMSF). The proteolytic products were resolved on 12% and 9% (w/v) SDS PAGE. One of each 12% and 9% gel were stained with Coomassie Blue and the other was transferred to a PVDF membrane and probed with the primary antibody, mouse anti HIS, and secondary HRP conjugated antibody goat anti mouse. Following detection using luminescence, the antibodies were stripped from the PVDF membrane, and the membrane was stained with Coomassie Blue. Selected bands were excised from the membrane and sent for N-terminal amino acid sequencing (Sheldon Biotechnology Centre, McGill University).

2.3.3. *DNA substrates*

Oligonucleotides labelled at the 5' end were generated by incubating upto 20 pmol dephosphorylated DNA with 20 pmol [γ -³²P]-ATP (Perkin Elmer) and 10 units of T₄ polynucleotide kinase (New England Biolabs) in the presence of 50 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA for 30 minutes at 37°C. Oligonucleotides labelled at the 3' end were generated by incubating 20 pmol of the annealed dsDNA substrate with 5 units of

Klenow exo^- (New England Biolabs) and 20 pmol of ($\alpha\text{-}^{32}\text{P}$)-GTP (Perkin Elmer) in the presence of 10 mM Tris pH 7.9, 10 mM MgCl_2 , 50 mM NaCl, and 1 mM DTT for 30 minutes at 37°C. Labelled oligonucleotides were purified using denaturing polyacrylamide (10%) gel electrophoresis (PAGE) composed of 7 M urea, 10% acrylamide and 1X TBE (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA). Double stranded substrates were generated by annealing labelled oligonucleotide with a molar equivalent of the non-labelled complementary oligonucleotide to produce blunt ends, 3' overhangs and 5' overhangs. Annealing was performed by heating to 95°C for 5 minutes followed by cooling to room temperature over 60 min. All of the DNA substrates labelled at the 3' end contained a free 5' phosphate unless stated otherwise. Quantification of DNA was performed by reading absorbance at 260 nm using $\epsilon = 50 \text{ ng-cm/mL}$ for dsDNA and 33 ng-cm/mL for ssDNA.

2.3.4. *In vitro* Nuclease Activity

Nuclease assays were performed by incubating varying concentrations of Pso2 with 1 pmol DNA in the presence of 10 mM Tris pH 7.9, 10 mM MgCl_2 , 50 mM NaCl, and 1 mM DTT in a final volume of 10 μL . Reactions were carried out at 25°C for various time intervals (0.25, 0.5, 0.75, 1, 2, 4, 8, 16 and 32 min) and quenched by addition of an equal volume of formamide and incubation at 90°C for 5 min. The DNA was resolved using 20% denaturing PAGE consisting of 7 M urea, 20% acrylamide and 1X TBE (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA) and analyzed by autoradiography.

Relative activities were determined using 5' labelled substrates containing a non-bridging phosphorothioate substitution between the second and third nucleotide (Alpha DNA) so that only a single reaction product could be generated for each DNA substrate. Radiolabelled DNA substrates and products were quantified using ImageQuant (Molecular Dynamics). All experiments were performed at least in triplicate.

Nuclease activity of *pso2* mutants was accessed by incubating 1 μ M protein with NdeI linearized pUC19 plasmid for 10, 20, 30, 40, 50, and 60 minutes. The DNA was analyzed on a 1% TBE agarose gel and visualized by ethidium bromide staining.

2.3.5. DNA binding assays

Increasing amounts of Pso2 were added to 4 pmol of 3' 32 P end-labelled ssDNA containing a phosphorothioate substitution between the first and second nucleotide and either a 5' phosphate or 5' hydroxyl. Pso2 is unable to digest DNA substrates containing a phosphorothioate substitution at the non-bridging oxygen located between the first and second nucleotides (Figure 2.1). Therefore, in order to analyze binding without the complication of substrate degradation, binding assays were performed using 5' hydroxyl or 5' phosphate containing substrates harboring a phosphorothioate substitution after the first nucleotide. The reactions were carried out in the presence of 10 mM Tris pH 7.9, 50 mM NaCl, 1 mM DTT, and 15% glycerol at 25°C for 20 min. The extent of DNA binding was analyzed by running reactions on an 8% non-denaturing gel in

TAE buffer (40 mM Tris, 40 mM acetic acid, 2 mM EDTA). Quantification of bound and unbound species was performed with ImageQuant (Molecular Dynamics).

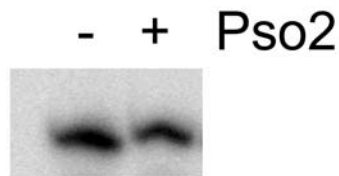


Figure 2.1 Pso2 exonuclease activity on ssDNA containing a phosphorothioate. DNA is 3' end labeled and contains a single phosphorothioate between the first and second nucleotides. No exonuclease activity was observed when wild type Pso2 was incubated with this substrate.

2.3.6. Determining Pso2 Processivity

The processivity of Pso2 was determined by incubating 0.5 pmol Pso2 with 2 pmol of 3' labelled single stranded substrate containing a 5' phosphate for 2 min at 25°C. Non-labelled competitor containing a 5' hydroxyl in increasing fold excess of 0.5, 1, 2, 4, 8, 16, 32, and 64 μ M was added. The 10 μ L reactions were incubated for an additional 10 min at room temperature in the presence of 10 mM Tris, pH 7.9, 10 mM MgCl_2 , 50 mM NaCl, and 1 mM DTT. The reactions were terminated and resolved as described previously. The percentage of product produced was determined by quantifying the intensity of the bands using ImageQuant (Molecular Dynamics).

2.3.7 Effects of phosphite addition on Pso2 nuclease activity

The effect of phosphite on activity was assessed by incubating 160 pmol Pso2 with increasing concentrations of phosphite of 8 μ M, 16 μ M, 32 μ M, 64 μ M, 125 μ M, 250 μ M, and 500 μ M. The reactions were incubated in the presence of 10 mM Tris pH 7.9, 10 mM MgCl₂, 50 mM NaCl, and 1 mM DTT for 10 min at 25°C prior to the addition of 1 pmol ssDNA labelled at the 3' end containing a 5' phosphate or hydroxyl. These 10 μ L reactions were incubated for an additional 2 min at room temperature, terminated, and resolved as described above.

2.3.8 Yeast survival assay

S. cerevisiae *pso2* null strains, complemented with pYES-DEST52 (Invitrogen) expression vectors containing WT or *pso2* point and deletion mutants, were grown to saturation at 30°C in minimal media lacking uracil containing raffinose as a carbon source. Cells were diluted to an optical density at 600 nm of 1.0 in drop out base media containing galactose as a carbon source. After adding cisplatin at increasing concentration (0.016 mM, 0.03 mM, 0.06mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1mM), cultures were grown at 30°C for an additional 2 hrs and then plated in serial dilution on minimal media lacking uracil for plasmid selection and containing galactose as a carbon source. Colony forming units were counted and compared to a control, prepared in the absence of cisplatin to assess percent survival. The experiment was performed in triplicate.

2.4. Results

2.4.1. Pso2 is a 5' phosphate-dependant 5'-exonuclease

Nuclease activity of highly purified Pso2 (Figure 2.2) was analyzed using a variety of DNA substrates. It has previously been reported that Pso2 is a 5'-exonuclease (Li, et al 2003). To begin our characterization, I first verified this activity. These experiments involved incubating increasing amounts of wild type Pso2 with a constant amount of a 12 nucleotide single-stranded DNA (ssDNA) substrate labelled with ^{32}P at the 5' end (Fig. 2.3 A). The presence of a single product corresponding to a single nucleotide confirmed that Pso2 acts as a 5'-exonuclease *in vitro*. I next examined whether Pso2 possesses a 3'-exonuclease activity. A 12-nucleotide DNA substrate ^{32}P -labeled at the 3' end was incubated with increasing amounts of Pso2. As shown in Figure 2.3 B, a full distribution of oligonucleotide products were generated, consistent with a 5' but not a 3'-exonuclease activity. The observed banding pattern also demonstrates that Pso2 is able to fully degrade the entire DNA substrate to a single mononucleotide indicating that Pso2 does not have a minimum DNA length requirement for activity.

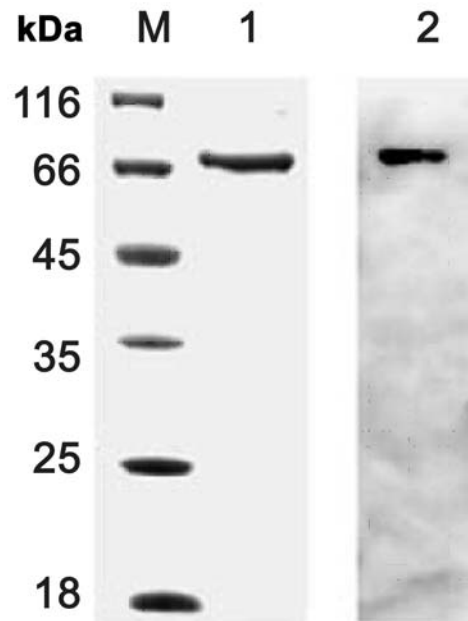


Figure 2.2 Purified Pso2. Full length wild type Pso2-HIS₆ migrates at 76 kDa on a 9% SDS PAGE gel. Lane 1, coomassie stain. Lane2, western blot of purified Pso2 using mouse α HIS primary and goat α mouse HPC secondary antibodies.

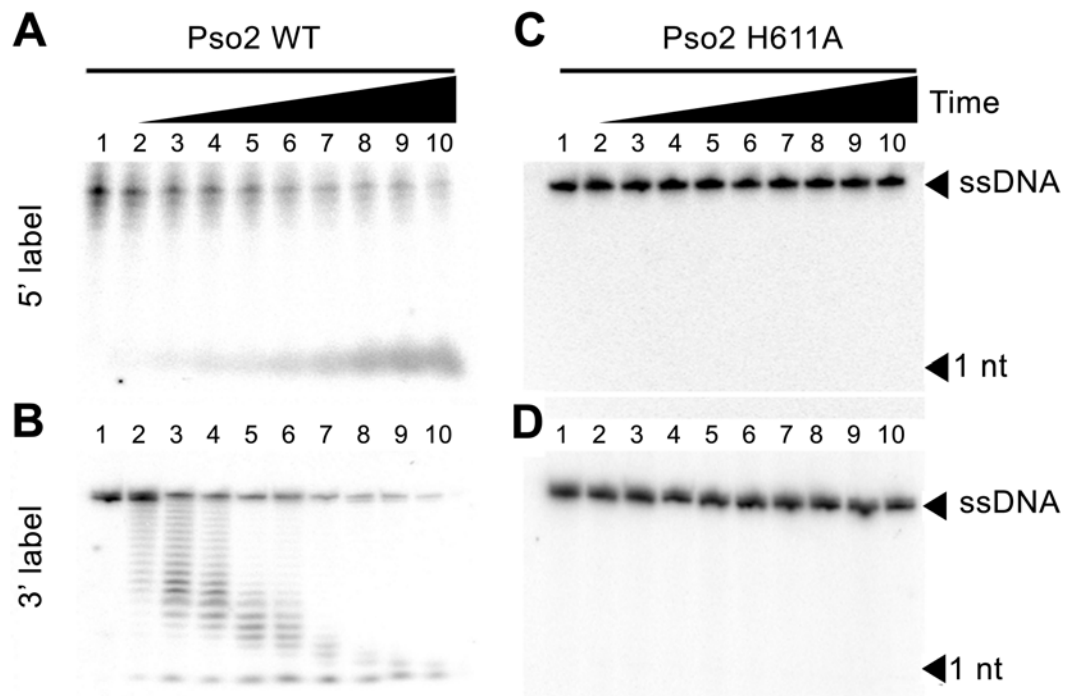


Figure 2.3 Pso2 possesses 5' but not 3' exonuclease activity. Pso2 (80 nM) was incubated with ssDNA (100 nM) labelled at the 5' (**A**) or 3' end (**B**). Purified *pso2* H611A (1 μ M) was assayed with ssDNA (100 nM) labelled at the 5' (**C**) or 3' end (**D**). Lane 1 in each panel contains no Pso2. Reactions in lanes 2–10 were carried out for increasing time intervals: 0.25, 0.5, 0.75, 1, 2, 4, 8, 16 and 32 minutes, respectively.

To confirm that the observed nuclease activity was due to Pso2 and not a contaminating nuclease the histidine at position 611 was substituted to alanine and the resulting Pso2 mutant tested for nuclease activity. Histidine 611 is conserved in all β -CASP domains and is proposed to coordinate a metal ion binding responsible for structural stability. *pso2* H611A displayed no nuclease activity towards either a 3' or 5' labelled substrate, confirming that the observed 5'-exonuclease activity is in fact due to Pso2 (Figure 2.3 C-D).

In these studies, while using 3' labelled DNA substrates it was observed that Pso2 was only able to degrade substrates that contained a free 5' phosphate group. As shown in Figure 2.4, identical ssDNA substrates differing only in the presence or absence of a 5' phosphate were tested as substrates for Pso2. Pso2 was able to fully degrade the substrate containing a 5' phosphate (Figure 2.4 B) but was unable to utilize the ssDNA substrate containing a 5' hydroxyl (Figure 2.4 A). These results demonstrate that the exonuclease activity of Pso2 is dependent on a 5' phosphate group.

The inability of Pso2 to degrade a 5' hydroxyl containing substrate could be attributed to an inability to bind substrates lacking a 5' phosphate. Electrophoretic mobility shift assays (EMSAs) were performed to determine whether Pso2 requires a 5' phosphate for DNA binding. Pso2 is unable to digest DNA substrates containing a phosphorothioate substitution at the non-bridging oxygen located between the first and second nucleotides (Figure 2.1). Therefore, in order to analyze binding without the complication of substrate

degradation, binding assays were performed using 5' hydroxyl or 5' phosphate containing substrates harboring a phosphorothioate substitution after the first nucleotide. In these assays, increasing amounts of Pso2 were incubated with either substrate and resolved under native conditions as shown in Figure 2.4 C. Pso2 was able to bind both substrates regardless of whether a 5' hydroxyl (Figure 2.4 C, lanes 2-4) or a 5' phosphate (Figure 2.4 C, lanes 6-8) was present. The dissociation constants for the 5' phosphate and 5' hydroxyl containing substrates were very similar at 8.0 ± 0.6 and 9.0 ± 4.0 nM, respectively. This result demonstrates that Pso2 is able to bind both substrates with a similar affinity. Collectively, these results indicate that the 5'-exonuclease activity of Pso2 is dependent on a 5' phosphate and that this dependence is not a result of binding affinity.

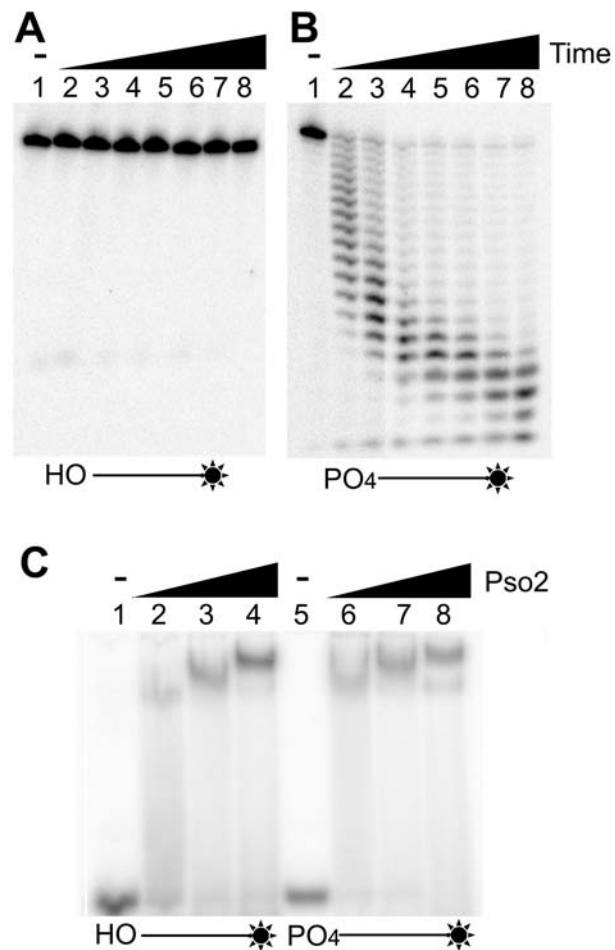


Figure 2.4 Pso2 requires a 5' phosphate for nucleotide removal. In panel (A) Pso2 (1.5 μ M) was assayed using 3' labelled 20 nt ssDNA (200 nM) containing a 5' hydroxyl. Lane 1 is a negative control in the absence of Pso2. Reactions in lanes 2–8 were incubated for 0.5, 1, 2, 3, 4, 8 and 16 minutes, respectively. Panel (B) is identical to panel (A) except substrate used contains a 5' phosphate. (C) EMSA reactions using increasing amounts of Pso2 were incubated with a 3' labelled 12 nt single stranded DNA substrate (100 nM) containing a phosphorothioate substitution between the first and second nucleotide as well as either a 5' phosphate or a 5' hydroxyl. Lane 1 and 5, no Pso2 added; lanes 2–4 contain 5' hydroxyl substrate with final Pso2 concentrations of 50, 100 and 200 nM, respectively. Lanes 6–8, 5' phosphate substrate with final Pso2 concentrations of 50, 100 and 200 nM, respectively.

2.4.2. Pso2 5'-exonuclease activity is not dependent on DNA structure

Pso2 is thought to function in ICL repair as a nuclease, processing one or more intermediates generated during a repair event. The precise role of Pso2 in ICL repair, however remains unclear. To help define a role(s) for Pso2, the ability of Pso2 to act upon different DNA structures generated during DNA repair events including ICL, was tested.

As shown in Figure 2.5 A-C, all double stranded DNA substrates (blunt ends, 3' and 5' overhangs, Table 1) were efficiently digested from the 5' end. However, similar to what was observed with ssDNA (Figure 2.3 A), if a 5'-OH was present, these DNA substrates were not degraded even when Pso2 was present at 10 fold higher concentration (Figure 2.5 D and E). Importantly, these results clearly demonstrate that Pso2 does not harbor endonuclease activity towards overhang structures (3' or 5'), ssDNA or dsDNA substrates.

Pso2 was further tested for its ability to gain entry and degrade DNA from a nicked substrate. A short 18 bp DNA substrate (Supplementary Table 1) with an internal nick and two hairpin ends was used for simplicity. As shown in Figure 2.5 F, Pso2 efficiently digested the substrate from the nick site in a 5' to 3' direction. In addition, Pso2 appeared to generate intermediates that are consistent with opening of hairpin structures on each end of the nicked substrate. Intermediates marked by pink and blue arrows in Figure 2.5 F indicate species that appear to have been produced by opening hairpins closest to the 5' and 3' ends, respectively. Both intermediates were further degraded by Pso2 due to the

presence of newly generated 5' phosphate groups upon hairpin opening. Thus, in addition to degrading DNA from a nick, Pso2 also generated intermediates consistent with an ability to open DNA hairpins.

The rate of Pso2 nuclease activity for each of the substrates analyzed is summarized in Table 1. These findings demonstrate that Pso2 is able to digest a wide range of DNA structures containing a free 5' phosphate, most of which represent intermediates formed during repair of ICLs and other forms of DNA damage. Given that only minor differences are observed in rates of Pso2 exonuclease activity (Table 1) it appears that Pso2 exhibits little DNA structure specificity, which is in marked contrast to the human homologue of Pso2, hSNM1A, which only degrades ssDNA.

Table 1 DNA substrates.

Oligo	Label	5'	Description	Sequence
1	5'	PO ₄	ssDNA	TTTTTTTTTTTTTTTTTTTT
2	3'	PO ₄	ssDNA	TTTTTTTTTTTTTTTTTTTTG
3	3'	OH	ssDNA	TTTTTTTTTTTTTTTTTTTTG
4	5'	PO ₄	ssDNA phosphorothioate	T*GGGGCCGCACA
5	3'	OH	ssDNA phosphorothioate	T*GGGGCCGCACAG
6	5'	PO ₄	Blunt (6+7)	TGGGGCCGCACA
7	None	OH	Blunt (6+7)	TGTGCGGCCCCA
8	5'	PO ₄	5' overhang (8+11)	GGCAGAGTGGGGCCGCACA
9	3'	OH	3' overhang (9+13)	GGCAGAGTGGGGCCGCACAG
10	None	OH	3' overhang (10+12)	GGCAGAGTGGGGCCGCACA
11	None	OH	5' overhang (8+11)	CTGTGCGGCCCC
12	5'	PO ₄	3' overhang (10+12)	GCCCCACTCTGCC
13	None	OH	3' overhang (9+13)	GCCCCACTCTGCC
14	3'	PO ₄	Nicked, double hairpin (14 only)	CTCAAGGGAACCTTGAGCAGAGGGGAACCCTCTG
15	5'	PO ₄	Closed, double hairpin (15 only)	CAATCAAGGGAACCTTGATTGCAGAGATGGGAACCATCTCTG
16	3'	OH	Single hairpin (16 only)	CAATCAAGGGAACCTTGATTG
17	3'	PO ₄	Crosslink (17+18)	CTTCCTTCTCCCTTCTCCTACTCTCCCTTCTCCCTCGCTCG
18	None	OH	Crosslink (17+18)	CGAGCGAGGGAGAAGGGAGAGTAGGAGAAGGGAGAAGGAAG

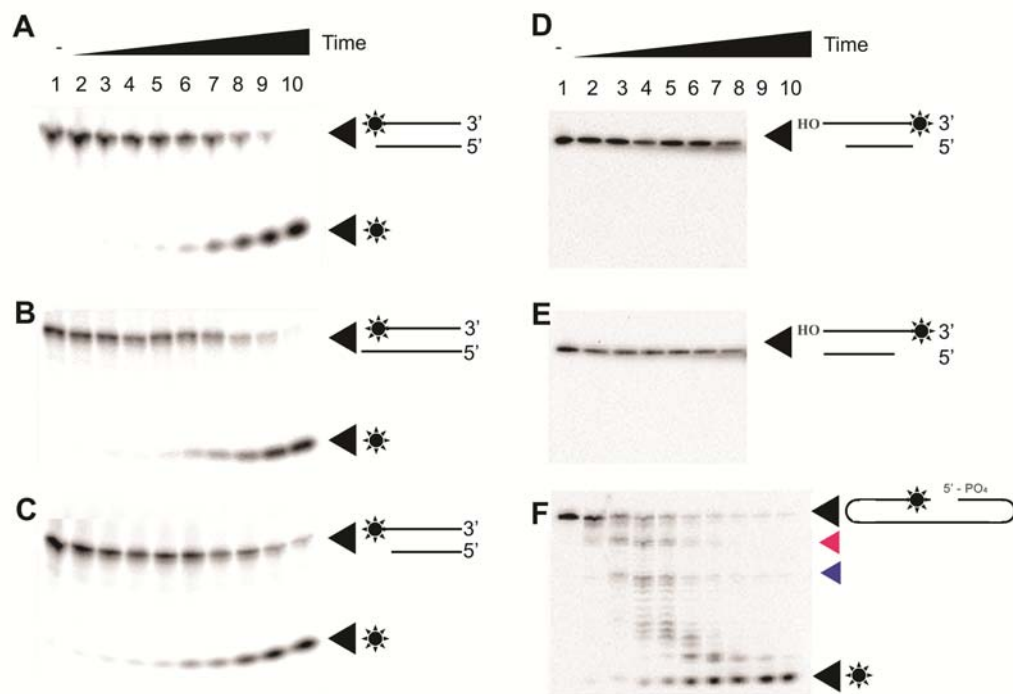


Figure 2.5 Pso2 exonuclease activity with DNA substrates containing 5' phosphates. In panels (A-C) Pso2 (80 nM) was assayed with 5' labelled double stranded DNA (100 nM) containing (A) blunt ends (B) 3' overhangs or (C) 5' overhangs. Lane 1 in each panel is a negative control where Pso2 was omitted. Lanes 2–10, reaction times: 0.25, 0.5, 0.75, 1, 2, 4, 8, 16 and 32 minutes, respectively. In panels (D-E) Pso2 (800 nM) was incubated with 3' labelled double stranded DNA (100 nM) containing (D) 3' overhangs or (E) 5' overhangs. In panel (F) Pso2 (80 nM) was assayed with 3' labelled double stranded DNA (100 nM) containing hairpin capped ends and a single nick. Lanes 2–7, reaction time: 1, 2, 4, 8, 16 and 32 minutes, respectively. Red and blue arrows indicate nicking of hairpins closest to the 5' and 3' ends, respectively.

Table 2 Pso2 nuclease activity with different DNA substrates.

DNA	k_{cat} (s^{-1})
Ss	0.018 ± 0.004
ds (blunt)	0.015 ± 0.0002
ds (3-overhang)	0.008 ± 0.006
ds (5-overhang)	0.005 ± 0.004
Hairpin	0.04 ± 0.01

2.4.3. *Phosphite inhibits Pso2 nuclease activity*

Given that a 5' phosphate was required for Pso2 exonuclease activity, an analysis was conducted to determine whether a mimic of this functional group could have an effect on activity. This analysis consisted of addition of phosphite to determine if it could stimulate exonuclease activity towards the substrate containing a hydroxyl group at the 5' end or alternatively inhibit exonuclease activity towards the substrate containing the 5' phosphate. Phosphite did not result in any stimulatory effect towards DNA containing a 5' hydroxyl group (Figure 2.6 A). However inhibition was observed towards the DNA substrate containing a 5' phosphate as indicated by decreased nucleotide degradation upon addition of increasing concentrations of phosphite (Figure 2.6 B). This suggests that the phosphite molecule may occupy the region of Pso2 responsible for 5'-phosphate interaction, therefore inhibiting the nuclease reaction.

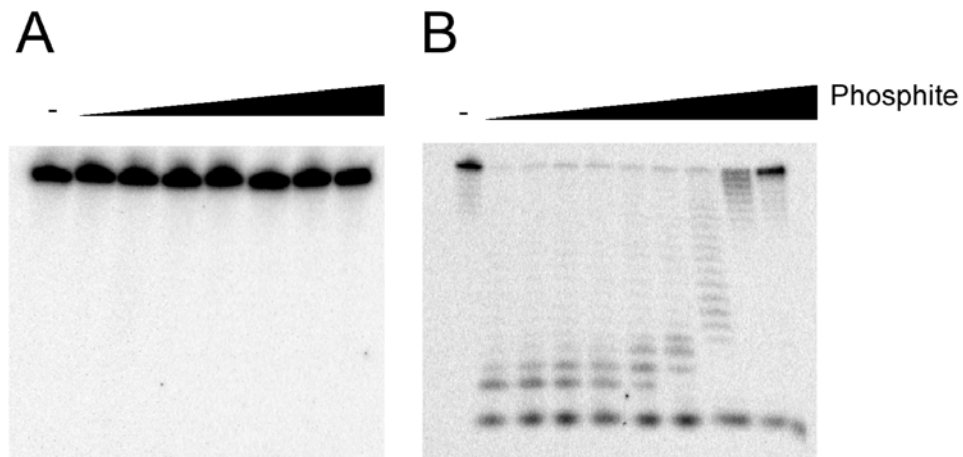


Figure 2.6 Pso2 is inhibited by phosphite. Pso2 (160 nM) was incubated with 8 μ M, 16 μ M, 32 μ M, 64 μ M, 125 μ M, 250 μ M, and 500 μ M of phosphite prior to addition of 100 nM of 3' labelled 20 nt DNA containing a (A) 5' hydroxyl or (B) 5' phosphate. Phosphite was not able to stimulate Pso2 activity towards substrate containing a 5' hydroxyl but did inhibit Pso2 activity towards a substrate containing a 5' phosphate.

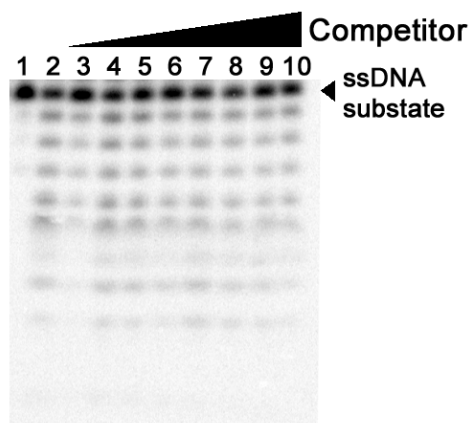


Figure 2.7 Pso2 is a processive exonuclease. Pso2 (0.5 μ M) was incubated with a 3' labelled 20 nt DNA substrate (4 μ M) containing a 5' phosphate, in the absence of Mg^{2+} . Non-labelled oligonucleotide containing 5' hydroxyl was added in increasing amounts along with 10 mM Mg^{2+} . Lane 1 indicates a negative control where Pso2 was omitted, lane 2 contains Pso2 and lacks the cold competitor DNA while lanes 3-10 cold substrate: labelled substrate in molar ratios of 1:8, 1:4, 1:2, 1:1, 2:1, 4:1, 8:1 and 16:1.

2.4.4. Pso2 is a processive exonuclease

Given the ability of Pso2 to efficiently degrade DNA to a single nucleotide, it would appear that Pso2 might function in a highly processive manner. The processivity of Pso2 was investigated by incubating a 3' labelled substrate containing a 5' phosphate with Pso2 in the absence of Mg^{2+} to allow for substrate binding but not hydrolysis. A non-hydrolysable substrate was then added in increasing concentrations as a cold competitor and reactions were initiated by addition of Mg^{2+} . In this experimental set-up, if Pso2 cleaves the first nucleotide and then releases the DNA prior to rebinding for removing the next nucleotide, addition of increasing amounts of cold competitor will titrate out Pso2. Such binding would reduce the degradation of DNA resulting in distinct banding patterns for reactions carried out in the presence and absence of competitor DNA. The data shown in Figure 2.7 illustrates that the degradation of the labelled DNA was similar in the absence and presence of cold competitor (Figure 2.7, compare lanes 2 with 3 - 10). This banding pattern suggests that Pso2 is a processive nuclease, remaining bound to the substrate throughout successive rounds of nucleotide removal.

2.4.5. The conserved β -CASP domain is required for *in vitro* nuclease activity and *in vivo* ICL repair

It has been previously demonstrated that the Pso2 amino acid substitution D252A, one of the conserved aspartic acid residues within the metallo- β -lactamase region of the β -CASP domain, results in reduced survival in the

presence of the crosslinking agents cisplatin and 8-MOP (Henriques & Moustacchi, 1980, Li & Moses, 2003). This residue resides within the metallo- β -lactamase domain and the alteration of the corresponding residue within Artemis also results in abolished *in vivo* and *in vitro* activity (Li & Moses, 2003, Li et al, 2005). Pso2 H611 resides within the CASP insertion of the β -CASP domain and amino acid substitution at this position (H611A) also displays complete loss of *in vitro* nuclease activity (Figure 2.3 C and D). To further characterize additional motifs and domains of Pso2 important for activity, several point and deletion mutations were constructed.

Pso2 deletions were generated based on results of limited proteolysis. N-terminal amino acid sequencing on limited protease stable domains of Pso2 revealed a protease sensitive domain spanning residues 1 - 188. Therefore, deletion mutants were constructed by truncating the N- and C-terminal domains, generating constructs 1-188 and 188-661. Point mutations were designed in order to disrupt the β -CASP domain (D231A and H611A) or zinc finger domain (C147A/C150A and C166A/C168A). The Pso2 mutant C147A/C150A was toxic in *E. coli* and therefore unable to be expressed; however, all other *pso2* mutants were expressed and purified (Figure 2.8).

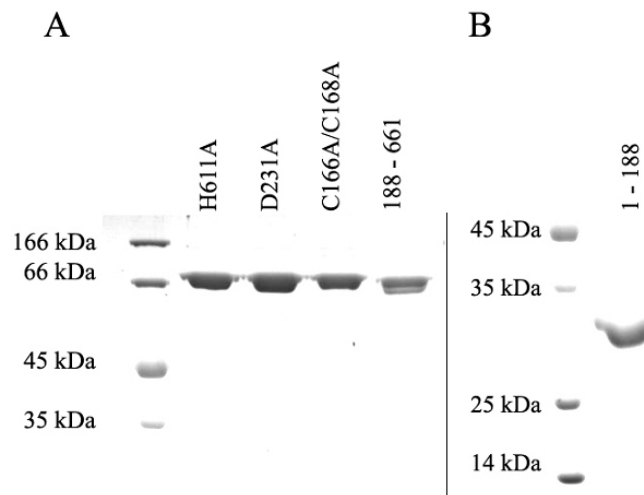


Figure 2.8 Purified *pso2* mutants. Coomassie stained gels of *pso2* H611A, D231A, C166A/C168A, 188-661, and 1-188 analyzed on a (A) 9% and (B) 12% PAGE.

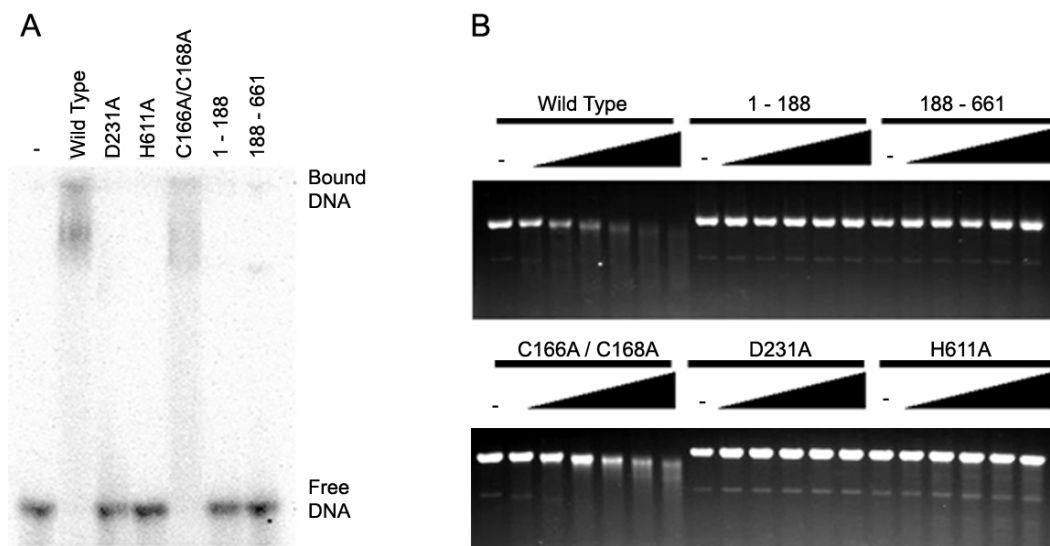


Figure 2.9 DNA binding and exonuclease activity of *pso2* mutants. (A) Pso2 WT and *pso2* mutants were incubated with 3' labelled 12 nt DNA. Shifting of DNA indicates DNA binding. (B) Pso2 WT and mutants (5 μ M) were incubated with linearized pUC19 plasmid. The disappearance of the DNA indicated exonuclease activity. *pso2* C166A/C168A retains both exonuclease and DNA binding capabilities while all other *pso2* mutants lack all activity.

The combined substitutions at C166 and C168 had no quantitative effect on DNA binding or exonuclease activity (Figure 2.9). The turnover for the double mutant (k_{cat} of $0.25 \text{ min}^{-1} \pm 0.03$) was indistinguishable from wild type levels (k_{cat} of $0.21 \text{ min}^{-1} \pm 0.02$). Nonetheless, qualitatively it appears that mutations to this zinc finger domain result in reduced exonuclease activity (Figure 2.9 B). This may be a result of processivity rather than initial removal of the first nucleotide. Disrupting the β -CASP domain via amino acid substitution of the conserved residues D231A or H611A; as well as the complete removal of the β -CASP or N-terminal domain abolished DNA binding and therefore exonuclease activity.

Deletion of Pso2 results in sensitivity toward cisplatin, as indicated by decreased survival, which is restored upon complementation with a wild type copy of Pso2 (Figure 2.10 A). To determine whether loss of *in vitro* activity, correlated to decreased levels of ICL survival, *pso2* mutants were tested for their ability to complement the *pso2* null strain. Deletion of the N- or C-termini, as well as amino acid substitution to the conserved residues D321 or H611, resulted in cisplatin sensitivity (Figure 2.10). Conversely, double amino acid substitution to C147/C150 or C166/C168 results in enhanced resistance of 6 and 13 fold above wild type levels, respectively.

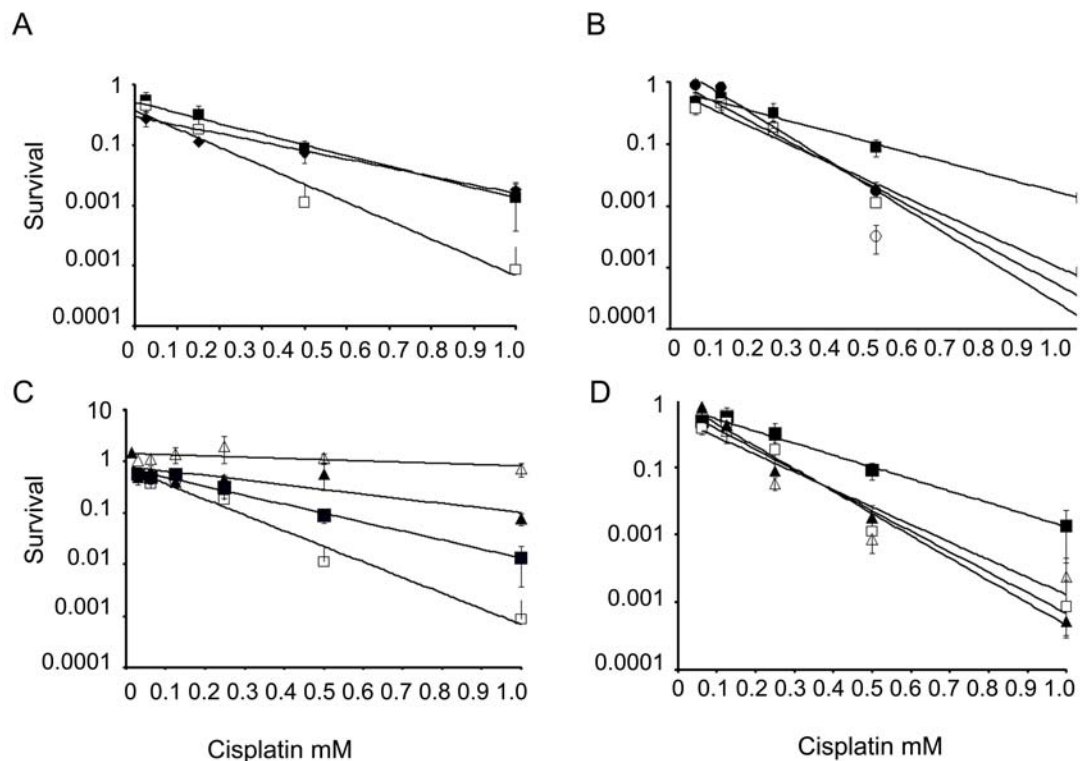


Figure 2.10 Survival of *Pso2* mutants in response to cisplatin. Survival after 2 hour incubation with increasing concentration of cisplatin for (A) wild type ($\diamond$), *Pso2* Δ ($\square$), and *Pso2* Δ + *Pso2* WT ($\blacksquare$); (B) *Pso2* Δ ($\square$), *Pso2* Δ + *Pso2* WT ($\blacksquare$), *Pso2* Δ + ΔC ($\circ$), *Pso2* Δ + ΔN ($\bullet$); (C) *Pso2* Δ ($\square$), *Pso2* Δ + *Pso2* WT ($\blacksquare$), *Pso2* Δ + C147A/C150A ($\blacktriangle$), *Pso2* Δ + C166A/C168A ($\diamond$); (D) *Pso2* Δ ($\square$), *Pso2* Δ + *Pso2* WT ($\blacksquare$), *Pso2* Δ + D231A ($\blacktriangle$), *Pso2* Δ + H611A ($\diamond$).

2.5. Discussion

Pso2 belongs to a small group of proteins required for repair of ICL induced damage (Pannicke et al, 2004). Of the 10 *PSO* genes characterized in *S. cerevisiae*, only *Pso2* is specifically required for repair of ICL lesions (Brendel et al, 2003a, Henriques et al, 1997). It has been shown that Pso2 (SNM1A) is essential for processing double strand breaks generated during repair of ICL damage, but not breaks generated by other means including exposure to bleomycin, HO endonuclease and ionizing radiation (Brendel et al, 2003b, Henriques et al, 1997). An obvious question then arises as to what DNA intermediate is generated during ICL repair that is unique from double strand breaks generated by other damaging events and that cannot be processed by nucleases functioning in other repair pathways. Despite being discovered more than 30 years ago, there are still no good mechanistic explanations for the specialized role of Pso2 in ICL repair. This work demonstrates that Pso2 displays processive 5'-exonuclease activity that is independent of DNA structure, but absolutely dependant on the presence of a 5'-phosphate.

2.5.1. *Pso2 is a 5' exonuclease with a dependence on a 5' phosphate*

The 5'-exonuclease activity of Pso2 was shown to be processive and dependent on the presence of a free 5' phosphate. This 5' phosphate requirement appears to be shared amongst other SNM1 proteins. Such a requirement is not common amongst most nucleases, although λ exonuclease, another highly processive 5'- exonuclease, does show a preference for substrates containing a free 5' phosphate. In the absence of a 5' phosphate, the activity of λ exonuclease decreases 10-fold (Henriques & Moustacchi, 1980, Li & Moses, 2003). The similar phosphate requirements observed between Pso2 and λ exonuclease suggest that these two proteins may share similarities in the mechanism through which they function. Interestingly, the results of a study examining the processivity of λ exonuclease demonstrated that the observed decrease in activity in the absence of a 5' phosphate is due to the formation of inert enzyme-substrate complex (Mitsis & Kwagh, 1999). Data presented here demonstrates that Pso2 is able to bind but not process DNA containing a 5' hydroxyl and is therefore consistent with the mechanism proposed for λ exonuclease. It is quite possible that other similarities between the two proteins exist, however a careful examination will be required before more correlations can be drawn.

This atypical phosphate dependence is unique and could potentially be exploited in the development of inhibitors targeted towards the human Pso2 homologue, SNM1A. Given that phosphite was able to inhibit Pso2 exonuclease

activity and that SNM1A shares the same 5' phosphate requirement, a small molecule could be developed towards SNM1A that resembles this structure. ICL inducing agents such as cisplatin are currently used in the treatment of tumorigenesis, however, like all DNA damaging agents used for chemotherapy, effectiveness is reduced by the function of endogenous repair pathways. Accordingly, resistance is often found to be linked to the up regulation of proteins involved in DNA repair (Mitsis & Kwagh, 1999). One strategy to combat this resistance is to inhibit the repair of DNA damage. SNM1A therefore represents an ideal target since it is specialized to the ICL repair pathway.

2.5.2. The β -CASP domain is required but not sufficient for Pso2 function

Mutations to conserved residues within the β -CASP domain completely abolished all *in vitro* exonuclease activity and *in vivo* ICL survival. Similarly, removal of the β -CASP domain or the N-terminal zinc finger domain resulted in loss of exonuclease activity and ICL resistance. This is in direct contrast to the human homologue Artemis in which the conserved residues have been shown to be non-essential for 5' exonuclease or DNA repair function (Aloyz et al, 2002, Chaney & Sancar, 1996). However, more recent investigations have demonstrated that exonuclease activity in these Artemis mutants may in fact be the result of contaminating nuclease activity (Pannicke et al, 2004). Interestingly, mutations to the zinc finger domain, which is not conserved amongst other β -CASP enzymes, resulted in an increase in ICL survival. This suggests that the N-terminal domain may serve as a regulatory function in Pso2. As an

exonuclease with little substrate preference such regulation may be essential for preventing unwarranted DNA degradation. This would account for the toxic effects observed in *E. coli* following over-expression of Pso2 lacking residues of the zinc finger domain. Artemis contains a C-terminal regulatory domain, which upon phosphorylation stimulates its nuclease activity. The complete removal of this C-terminal domain from Artemis relieves the suppression and results in constitutive activity (Pawelczak & Turchi, 2010). Likewise, substitution of cysteine residues of Pso2 zinc finger domain may also result in a constitutively active nuclease. In this case one might expect enhanced ICL repair as observed. Additionally it would explain the toxic effects observed during overexpression of this construct.

2.6. Conclusion

In the work presented here a thorough biochemical characterization of the DNA repair protein Pso2 has been provided. This 5'-exonuclease requires the presence of a free 5' phosphate for enzymatic activity but not DNA binding. In addition, Pso2 was shown to be a highly processive exonuclease that degrades its substrates to single nucleotides. Analysis of different DNA substrates revealed that Pso2 is able to process a variety of DNA substrates containing; 5' overhangs, 3' overhangs, and nicks. Many of these substrates may represent intermediates encountered during ICL damage or repair. However, it remains unclear why this specialized 5' exonuclease would be recruited specifically for ICL repair events, given that several exonucleases exist in the cell. Since Pso2 has no known additional roles in the cell it seems unlikely that its sole function would be limited to 5' exonuclease activity following ICL exposure. More probable is that either Pso2 is required for alternative cellular functions and is recruited to ICL damage or that Pso2 has other activities that are required for ICL repair aside from 5' exonuclease activity. To address this question further analysis of Pso2 must be conducted using more complex substrates both *in vitro* and *in vivo*; in particular substrates containing an internal interstrand crosslink and a DNA hairpin.

CHAPTER THREE

CHARACTERIZATION OF POSSIBLE INTERMEDIATES IN INTERSTRAND CROSSLINKING REPAIR REVEALS PSO2 IS CAPABLE OF OPENING DNA HAIRPINS

3.1. Abstract

Many types of DNA structures are generated in response to DNA damage, repair and recombination that require processing via specialized nucleases. One interesting class of structures are DNA hairpins formed during V(D)J recombination, palindrome extrusion, DNA transposition, and some types of double strand breaks. Here biochemical and genetic evidence is presented to suggest that Pso2 is the dominant DNA hairpin opening activity in budding yeast. Pso2 (SNM1A in mammals) belongs to a small group of proteins thought to function predominantly during interstrand crosslink (ICL) repair. In the previous section, the nuclease activity of Pso2 was characterized toward a variety of DNA substrates. In addition to non-specific 5' exonuclease activity, Pso2 appeared to also have DNA hairpin opening activity. In the work here, we show that Pso2 is an efficient, structure-specific DNA hairpin opening endonuclease. This activity was further shown to be required *in vivo* for repair of chromosomal breaks harboring closed hairpin ends. These findings provide the first evidence for Pso2 functioning outside ICL repair and open the possibility that Pso2 may function at least in part during ICL repair by processing DNA intermediates including DNA hairpins or hairpin-like structures.

3.2. Introduction

DNA hairpins pose a significant challenge to genome stability. Once formed, such structures must be removed and/or opened by specialized nucleases to permit subsequent repair. DNA hairpins are generated in a variety of ways with perhaps the most well characterized being programmed hairpin formation resulting from V(D)J recombination. DNA hairpins are also formed as a direct consequence of DNA damage and in some instances as intermediates during DNA repair. Regardless of how DNA hairpins are generated, these ends must be appropriately processed before ligation can occur. Failure to do so results in accumulation of double strand breaks, and thus a potentially lethal cellular event.

Human cells maintain at least two mechanisms for removal of DNA hairpins. The first involves Mre11, a well characterized nuclease essential for double strand break (DSB) repair. In conjunction with Sae2, Mre11 catalyzes the entire removal of DNA hairpin structures (chapter 1, Figure 1.5). Mre11/Sae2 function at damaged cruciform structures that have been converted into DNA hairpins with a single strand nick distal to the hairpin cap (Niewolik et al, 2006). Mre11 opens a gap from the available nick using its 3'-exonuclease activity and stimulates Sae2 to cleave the resulting ssDNA on the opposing strand, thereby completely removing the hairpin structure. Artemis (SNM1C) represents a second specialized nuclease capable of processing DNA hairpins; however, its function appears to be largely restricted to repair associated with the non-

homologous end-joining (NHEJ) DSB repair pathway following V(D)J recombination. The structure-specific endonuclease activity of Artemis (SNM1C) generates nicks at or very close to the apex of DNA hairpin structures, generating free ends for further processing and repair (Lengsfeld et al, 2007).

While investigating Pso2 5' exonuclease activity an interesting observation suggested possible DNA hairpin endonuclease activity. Given that the human homologue SNM1C (Artemis) also possesses DNA hairpin opening it is likely that yeast Pso2, containing the same β -CASP domain, would also have the ability to open DNA hairpins. Additionally, this activity would be unique amongst proteins involved in ICL repair and would explain the requirement of this specialized nuclease.

Work presented here demonstrates that Pso2 functions as an efficient DNA-structure specific hairpin opening endonuclease *in vitro*. We also show that Pso2 is required for repair of DNA hairpin structures in yeast. Together these results suggest that Pso2 may function in ICL repair by processing intermediates that have a hairpin or hairpin like structure. There is no known Artemis function in yeast. Our data suggests that Pso2 may represent the yeast homologue, possessing similar nuclease activities.

3.3. Materials and Methods

3.3.1. DNA substrates

Oligonucleotides labelled at the 5' end by incubating 20 pmol dephosphorylated DNA with 20 pmol [γ - 32 P]-ATP (Perkin Elmer) and 10 units of T₄ polynucleotide kinase (New England Biolabs) in the presence of 50 mM Tris, pH 7.6, 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine and 0.1 mM EDTA for 30 minutes at 37°C (New England BioLabs). Oligonucleotides labelled at the 3' end were generated by incubating 20 pmol of the annealed dsDNA substrate with 5 units of Klenow exo⁻ (New England Biolabs) and 20 pmol of (α - 32 P)-GTP (Perkin Elmer) in the presence of 10 mM Tris pH 7.9, 10 mM MgCl₂, 50 mM NaCl, and 1 mM DTT for 30 minutes at 37°C. Labelled oligonucleotides were purified using denaturing polyacrylamide (10%) gel electrophoresis (PAGE). Double stranded substrates were generated by annealing labelled oligonucleotide with a molar equivalent of the non-labelled complementary oligonucleotide to produce blunt ends, 3' overhangs and 5' overhangs. Annealing was performed by heating to 95°C for 5 minutes followed by cooling to room temperature over 60 min. All of the DNA substrates labelled at the 3' end contained a free 5' phosphate unless stated otherwise. Specific oligonucleotide sequences used in substrate generation are listed in Table 1 (Chapter 2).

Double stranded DNA substrate containing two hairpin capped ends was generated by 5' labeling of the sequence 5' –CAATCAAGGGAACCTTGATTG CAGAGATGGGAACCATCTCTG, prior to ligation with T4 ligase in the presence

of 40 mM Tris-HCl pH 7.8, 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP at 25°C overnight. The integrity of this substrate was verified by treatment with T7 exonuclease.

DNA substrate containing an interstrand crosslink was prepared using the method of K. Chválová *et al* 2007, by treating cisplatin (CAS 15663-21-1) with an equimolar concentration of AgNO₃ and removing the precipitate via centrifugation. Four molar equivalents of activated cisplatin were incubated with one molar equivalent of the oligonucleotide 5' CTCCTTCTCCCTTCTCCTACT CTCCCTTCTCCCTCGCTCT, containing a 3' label and 5' phosphate, in 100 mM sodium perchlorate pH 5.6 at 37°C for 7 days in the dark. The oligonucleotide containing a monoadduct at G was gel purified and annealed with the complementary strand in the presence of 400 mM NaCl. The dsDNA was exchanged into 100 mM sodium perchlorate pH 5.6 and incubated at 37°C for 14 days in the dark. Quantification of DNA was performed by reading absorbance at 260 nm using $\epsilon = 50$ ng-cm/mL for dsDNA and 33 ng-cm/mL for ssDNA.

3.3.2. *In vitro* Nuclease Activity

Nuclease assays were performed by incubating varying concentrations of Pso2 with 1 pmol of ³²P labeled DNA in the presence of 10 mM Tris pH 7.9, 10 mM MgCl₂, 50 mM NaCl, and 1 mM DTT in a final volume of 10 µL. Reactions were carried out at 25°C for various time intervals (0.25, 0.5, 0.75, 1, 2, 4, 8, 16 and 32 min) and quenched by addition of an equal volume of formamide and incubation at 90°C for 5 min. The DNA was resolved using 20% denaturing

PAGE consisting of 7 M urea, 20% acrylamide and 1X TBE (89 mM Tris base, 89 mM Boric acid, 2 mM EDTA) and analyzed by autoradiography.

3.3.3. *In vivo* Endonuclease Activity

S.cerevisiae MYO37 was generously provided by Cliff Weil (Department of Agronomy, Purdue University) and transformed with pWL201 (Ma et al, 2002). These cells were grown to saturation at 30°C in drop out base media containing raffinose as a carbon source. Induction of transposase was carried out by the addition of 1% galactose. Cells were allowed to grow at 30°C overnight prior to serial dilution and plating onto media lacking uracil or both uracil and adenine. Transposition frequency was determined by dividing the number of *ade*⁺ revertants by the total number of colony forming units.

3.4. Results

3.4.1 *Pso2* is not a translesional exonuclease

Pso2 represents one of only a small number of proteins currently thought to function solely in repair of interstrand crosslinks (Yu et al, 2004). The possibility that Pso2 might function as a ‘translesional’ exonuclease by removing one strand of an ICL lesion, unimpeded by the presence of a crosslink, was tested. However, when incubated with a DNA substrate containing a single interstrand crosslink, Pso2 was unable to digest past the ICL lesion (Figure 3.1), suggesting its role in ICL repair is not associated with ‘translesional’ 5’ exonuclease function.

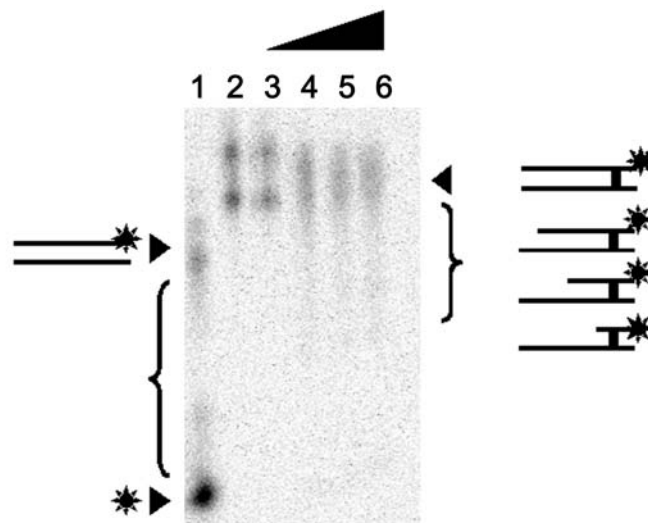


Figure 3.1 Pso2 is not a translesion exonuclease. Pso2 (1 μ M) was incubated with a dsDNA substrate containing no ICLs (lane 1) and containing a single ICL (lanes 2 - 6). Lane 2, no Pso2; lanes 3 – 6, Pso2 incubation with increasing time 15, 30, 60, 90 min, respectively. Pso2 fully degrades a substrate lacking an ICL. In the presence of an ICL, the DNA substrate remains much larger indicating that Pso2 exonuclease activity is blocked by the presence on an ICL.

3.4.2. *Pso2 is a structure specific endonuclease*

Results obtained during the analysis of Pso2 exonuclease activity with a nicked DNA substrate (Chapter 2; Figure 2.5 F), suggested that Pso2 may be able to open DNA hairpin structures. To verify this activity, Pso2 was analyzed against three substrates containing DNA hairpins. The first consisted of two identical plasmids differing only by the insertion of a short sequence that forms a protruding cruciform structure. Pso2 was able to linearize the hairpin containing plasmid presumably by opening these juxtaposed hairpins (Figure 3.2). The linear plasmid was further digested by Pso2 5' exonuclease activity.

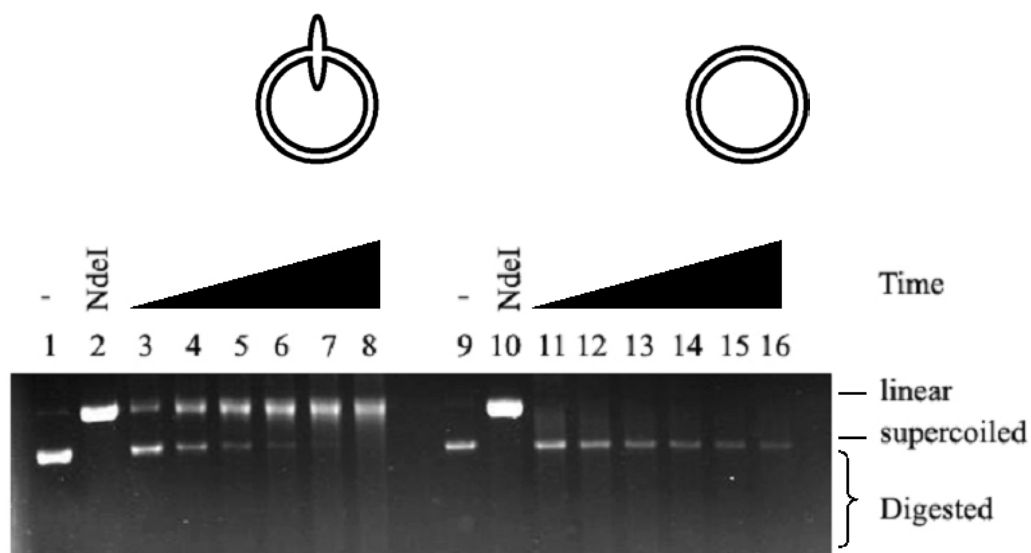


Figure 3.2 Pso2 opens DNA hairpin on plasmid DNA. Pso2 (5 μ M) was assayed pUC(AT), lanes 1-8 or pUC19, lanes 9-16. Lane 1 and 9 in each panel is a negative control in the absence of enzyme, lane 2 and 10 is the plasmid linearized with NdeI and lanes 3–8 and 11-16 contain Pso2. Endonuclease activity is observed by the loss of supercoiled DNA and the appearance of linear DNA. The linear DNA becomes a substrate for further degradation.

This activity was verified by testing the ability for Pso2 to process a doubly hairpined substrate containing no nicks. The integrity of the fully closed hairpin structure was confirmed by treatment with both ssDNA and dsDNA exonucleases (RecJf and T7) (Figure 3.3). As shown in Figure 3.3, Pso2 was able to efficiently open DNA hairpin structures. In fact the rate of hairpin opening exceeded exonuclease activity by ~2 fold (Table 1). Mutants lacking exonuclease activity (H611A, D231A, *pso2*₁₋₁₈₈, *pso2*₁₈₈₋₆₆₁, Figure 2.9) failed to open hairpin structures (Figure 3.4) while *pso2* C166A/C168A retained wild type levels of both 5'-exonuclease and DNA hairpin opening endonuclease activity, suggesting that Pso2 makes use of a single active site for both nuclease activities. Taken together with the fact that endonuclease activity was not observed for linear ssDNA or dsDNA substrates (Chapter 2), this data strongly suggests that Pso2 is a structure-specific endonuclease.

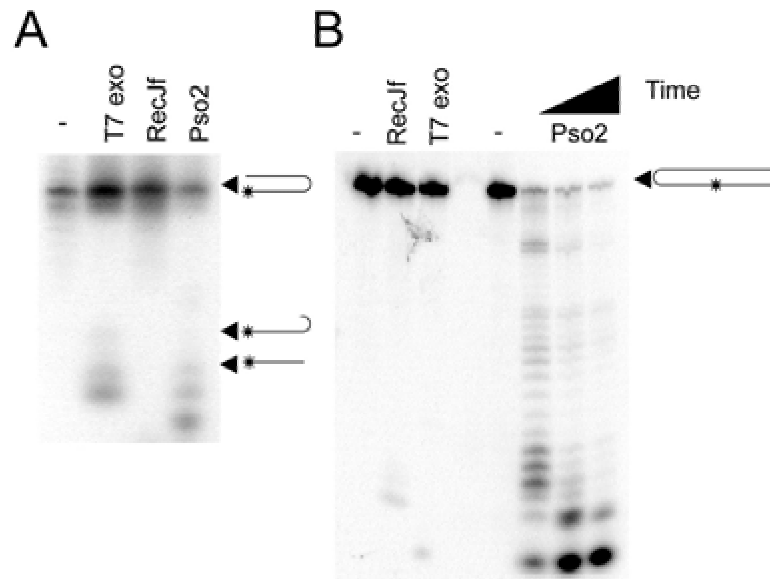


Figure 3.3 Confirmation of hairpin structure. DNA substrates containing either a single hairpin **(A)** or covalently closed double hairpin **(B)** were interrogated with T7 ds- and RecJf ss-exonuclease activities to verify structural integrity of each substrate. In **(A)** T7 ds-exonuclease is able to degrade the hairpin to half its original size, while RecJf had no effect since there is no ssDNA present in this substrate. Pso2 opens the hairpin on the 3' side of the apex. In **(B)** neither RecJf or T7 exonuclease activities were able to degrade the fully closed double hairpin structure. Pso2 opened the hairpin structures and subsequently degraded the substrate using its 5'-exonuclease activity.

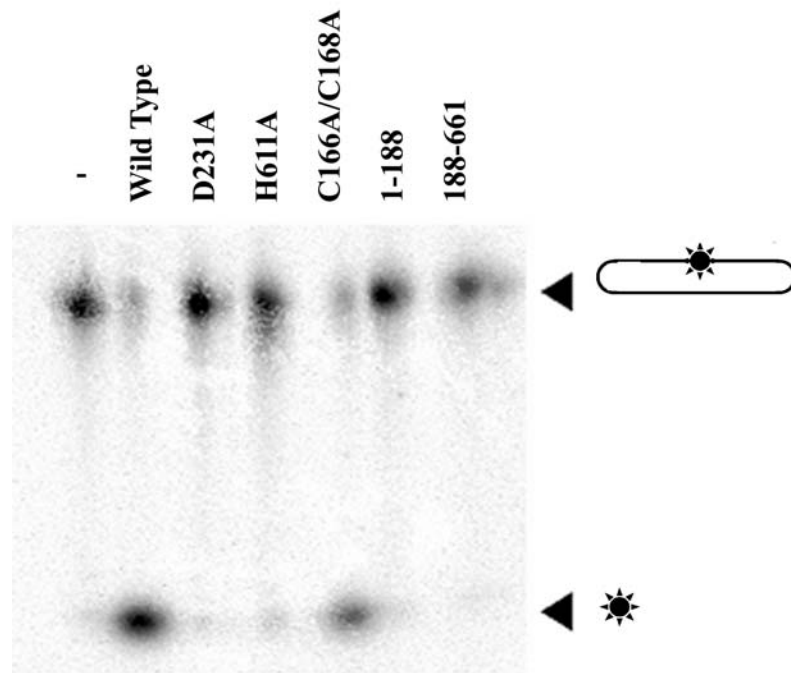


Figure 3.4 Endonuclease of *Pso2* mutants. *Pso2* WT and *pso2* mutants (1 μ M) were incubated with a completely closed hairpin substrate comprised of 20 bp duplex, two 3 nt DNA hairpins at each end and an internal label. Only wild type and *pso2* C166A/C168A show endonuclease hairpin opening activity.

To determine the exact location of DNA strand cleavage kinetic analyses were performed for Pso2 endonuclease activity using a 3'-³²P labelled hairpin substrate lacking a 5'-PO₄, since this substrate is only susceptible to Pso2 exonuclease activity once the hairpin has been opened. The fidelity of this substrate was established via treatment with T7 and RecJf exonuclease activities. As expected, T7 exonuclease degraded the substrate to half its original size while RecJf had no effect (Figure 3.3). Kinetic analysis of endo- and exonuclease function with this substrate (Figure 3.5) demonstrated the ability of Pso2 to open the hairpin on the 3' side of the apex and then remove single nucleotides from the exposed 5'-PO₄. The preferred site for cleavage occurred two nucleotides away from the hairpin apex.

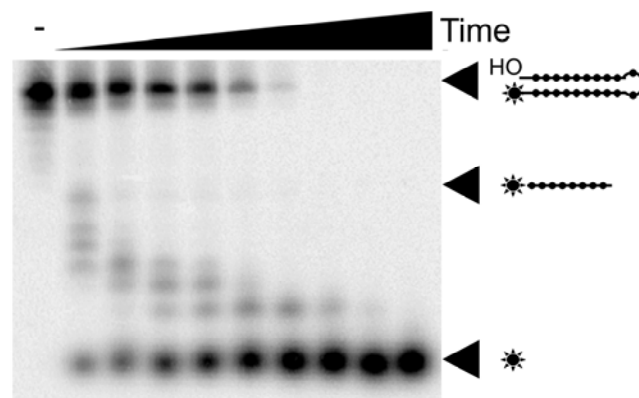


Figure 3.5 Kinetic analysis of Pso2 endonuclease activity. Kinetic analysis of Pso2 (80 nM) endonuclease activity was performed with a 3' labelled DNA hairpin containing 9 bp of dsDNA, a single 3 nt hairpin and 5'-OH (100 nM). An 8 nt product is generated immediately following initiation of the reaction that is consistent with hairpin opening 2 nucleotides from the apex on the 3' side.

3.4.3. *Pso2* is the dominant DNA hairpin opening activity in *S. cerevisiae*

Identification of a robust Pso2 hairpin opening activity *in vitro* suggested that hairpin intermediates may be encountered and processed by Pso2 during DNA repair *in vivo*. To further investigate this possibility, Pso2 was analyzed for the ability to open chromosomal hairpin structures in an *in vivo* transposon based assay.

In this assay, an *ADE2* gene is disrupted by insertion of an Ac/Ds transposon rendering cells incapable of growth in the absence of adenine. Ac/Ds is a member of the hAT superfamily of transposons that generate hairpin structures in the flanking chromosomal sequence during transposon excision (Weil and Kunze, 2000; Zhou et al, 2004). Thus, upon induction of transposase, Ac/Ds is excised leaving DNA hairpins structures in the flanking sequence. Removal of these hairpins is required for both cell survival and reversion to an ADE⁺ phenotype (Yu et al, 2004). Hairpin repair was monitored by growth on drop out base media following induction of transposon excision. As expected, reversion was reduced when *pso2* was deleted. Complementation with *Pso2* restored the rate of ADE⁺ revertants to wildtype levels (~100 fold) suggesting that Pso2 nuclease function was responsible for repair of hairpin structures in > 95% of recovered revertants (Figure 3.6). The remaining revertants, recovered in the absence of Pso2 (Figure 3.5, bar 2), are presumably processed by the well characterized hairpin opening activities of MRX and Sae2 complexes (Yu et al, 2004). Interestingly, when Mre11 and Sae2 were deleted revertants were unable

to be recovered, suggesting these proteins play an important role in the repair process (Figure 3.7). However, since Mre11 and Sae2 are important for end joining (Lengsfeld et al, 2007, Paull & Gellert, 1998), Lee and Lee 2007) it is difficult to assess their direct contributions in hairpin removal, as the ability to recover revertants in this assay is absolutely dependent on functional end joining. Mutants unable to restore ICL resistance in a *pso2* deficient strain (Chapter 2) were also unable to restore DNA hairpin opening in this strain with significant P values. (Figure 3.6). Mutations within the zinc finger domain (C166A/C168A) resulted in ADE⁺ revertants reduced compared to wild type levels (Figure 3.6).

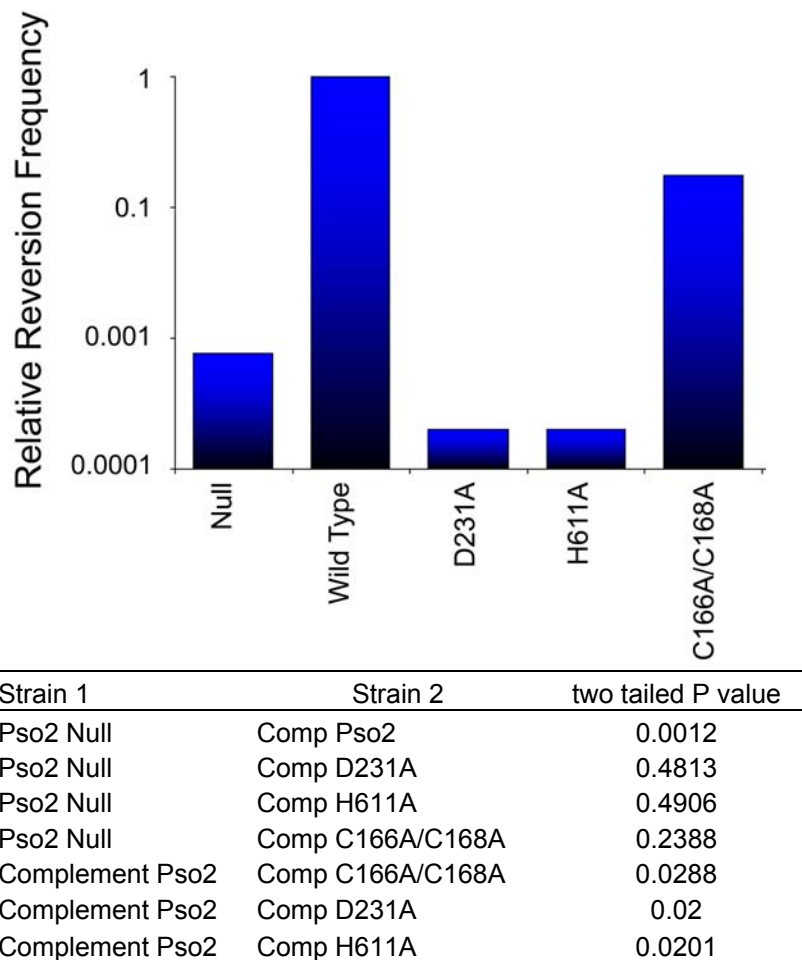


Figure 3.6 *In vivo* analysis of Pso2 hairpin opening activity. Reversion frequency, normalized to wild type is plotted as a function of Pso2 status in *S. cerevisiae*. Reversion frequency correlates with ability to repair chromosomal DNA hairpins generated via excision of the Ac transposon from within the *ADE* gene. *Pso2* null strains have decreased repair, which is only restored by complementation with a wild type copy or *pso2* C166A/C168A. Experiments were performed in at least triplicate.

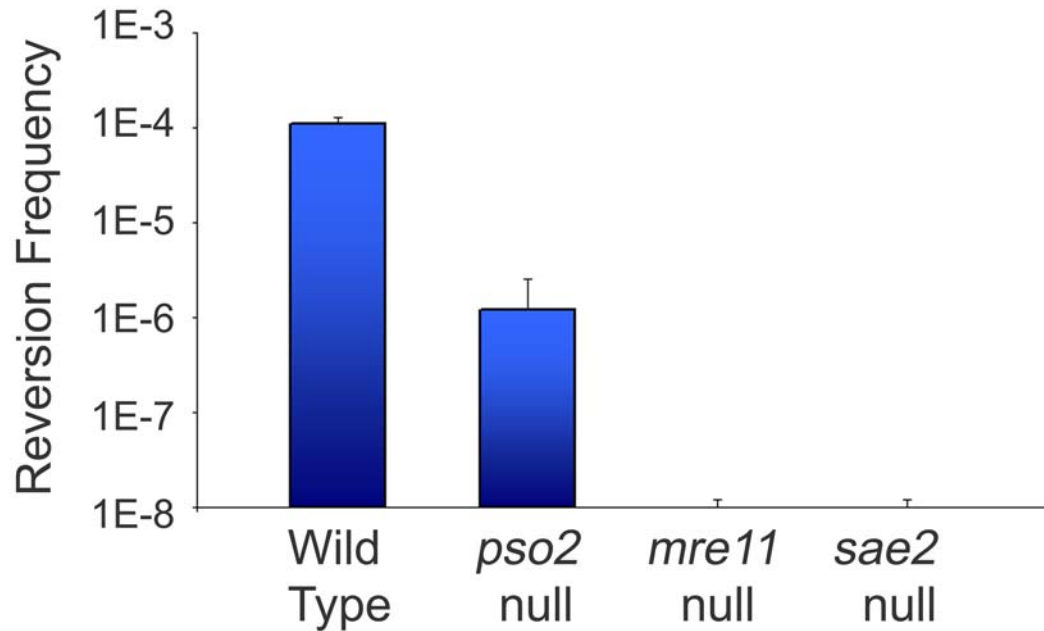


Figure 3.7 Comparison of DNA double strand break repair frequencies. The ability to repair DNA double strand breaks generated via transposon excision was determined by the frequency of reversion from ADE⁻ to ADE⁺. *mre11* and *sae2* deletions were unable to restore ADE function, indicating an inability to repair double strand breaks. Error bars represent standard deviation from experiments performed in at least triplicate.

3.5. Discussion

Pso2 possesses a robust hairpin-specific endonuclease activity. This is clearly demonstrated by *in vitro* analysis where only wild type catalytically active *pso2* mutants were able to open DNA hairpin structures. Hairpin opening activity was also observed *in vivo* where Pso2 was required to repair double strand break containing capped DNA hairpin ends in *S. cerevisiae*. Pso2 is not required for repair of double strand breaks generated by other means. The only difference between these breaks is the presence of DNA hairpins. At what point such hairpins might occur in budding yeast is not clear; however, this work suggests that if formed, Pso2 would be expected to contribute to their resolution and repair. Together, these results identify several novel aspects of Pso2 activity, and raise questions regarding the mechanism of Pso2 (SNM1A) in repair of DNA intermediates generated either directly or indirectly through exposure to ICL inducing agents.

3.5.1. Role of Pso2 in DNA hairpin opening

DNA hairpins represent a severe threat to cell viability. Such structures are known to occur in the cell and must be removed when located at the ends of chromosomal double strand breaks (reviewed in Lobachev et al, 2007). Failure to properly remove such structures results in aberrant replication, chromosomal rearrangements, DSB formation and ultimately cell death (Stark et al, 1989; Gardening et al, 1998). Cells utilize at least two mechanisms for hairpin processing. The first involves hairpin ‘removal’ and is proposed to make use of a

nick close to the hairpin end; (Huang & Dynan, 2002, Moore & Haber, 1996) while the second involves ‘opening’ of the hairpin close to its apex and is independent of a nick (Lengsfeld et al, 2007) (Chapter 1, Figure 1.5). In mammalian cells, Artemis is essential for opening of fully paired hairpin structures generated by RAG1/2 during V(D)J recombination (Lobachev et al, 2002, Ma et al, 2002). In the absence of Artemis, hairpin opening is severely reduced at coding joint ends suggesting that other hairpin opening endonuclease functions, such as the MRX (MRN in mammals) complex are unable to efficiently recognize and/or open these hairpin structures (Ma et al, 2002). Closely related human homologues of Artemis (SNM1A and Apollo) do not appear to be able to open these DNA hairpins either. In both yeast and mammalian cells, the MRX (MRN) complex has been shown to mediate processing of hairpin structures generated at long inverted repeat sequences (Rooney et al, 2002b, Farah et al, 2005). It is unclear why these proteins are unable to efficiently open the hairpin structures generated during V(D)J recombination. One possible explanation is that the hairpin substrates opened by Artemis and the MRN complex are structurally different. In support of this idea, MRN *in vitro* endonuclease activity is increasingly reduced as the size of the unpaired hairpin tip is decreased (Lobachev et al, 2002). Since V(D)J generated hairpins are fully paired it seems likely that MRN would not be able to efficiently act upon these hairpins. Hairpins created in this study closely mimic those generated during V(D)J recombination. The only homologue of Artemis in *S. cerevisiae* is Pso2 suggesting that Pso2

may fulfill a similar function to Artemis in yeast. This similarity in endonuclease activity may be related to the 5'-phosphate dependence observed for both of these proteins' 5'-exonuclease activities *in vitro*. In further support of this idea, both Pso2 and Artemis cleave DNA hairpin structures two nucleotides from the hairpin apex on the 3' side underscoring the similar manner in which they engage and process their substrates. Human Pso2 (SNM1A) has not been found to open hairpins *in vitro*; however, like Artemis (SNM1C) this function may be tightly regulated and only apparent *in vitro* upon appropriate stimulation (Paull & Gellert, 1998). Given the close phylogenetic relationship between Pso2 and SNM1A, and their shared roles in ICL repair, it seems possible that human SNM1A may also have endonuclease activity similar to Artemis that is only manifest upon appropriate post-translational modification.

In *pso2* deficient cells, rejoining of fully paired hairpin ends is severely impaired, nonetheless not completely abolished. This phenomenon is also observed in Artemis deficient cells suggesting that other cellular processes are able to open these DNA hairpins in the absence of Pso2 or Artemis albeit at much lower frequencies (Hejna et al, 2007). The most likely candidate for this repair is the MRX/N complex. There is good evidence suggesting that Pso2 and MRX may function on related DNA intermediates *in vivo*. Under conditions where cruciform structures are converted to hairpin capped double stranded breaks, a growth defect is observed in *mre11-H125N* strains that is not observed in *pso2Δ* strains (Rooney et al, 2002b). When both *pso2* and *mre11* are

deficient, there is a further 10-fold decrease in growth, indicating that in the absence of Mre11, Pso2 is involved in repairing similar DNA intermediates. Importantly, this sensitivity does not occur when damage is generated by HO-endonuclease further suggesting that Pso2 and Mre11 non-epitasis is limited to activity toward related hairpin structures.

3.6. Conclusion

This work demonstrates that Pso2 is required for processing DNA hairpins not readily accessed by other nucleases. This phenomenon is apparent *in vivo* and is independent of ICL damage, suggesting that Pso2 may play a cellular role in DNA hairpin processing that is in addition to its specialized function in ICL repair. Furthermore, Pso2 was shown to account for > 95% of the fully paired hairpin opening activity in *S. cerevisiae*, explaining its non-epistatic relationship to MRX (Lam et al, 2008). Although both these functions process hairpins, they do not appear to work equally well on precisely the same intermediate. While Pso2 efficiently opens fully paired hairpin structures its activity on hairpin structures generated at long inverted repeats is apparently greatly reduced compared to the MRX complex (Lobachev et al, 2002). It will be particularly interesting to determine if the novel hairpin opening activity of Pso2 is important for its specialized role in ICL repair. If so, it is possible that the human homologue of Pso2 (SNM1A) may also be involved in processing similar DNA intermediates. Given that unregulated nuclease activity is associated with cell

toxicity, SNM1A endonuclease activity would be expected to be highly regulated in mammals. Therefore like its orthologue Artemis, SNM1A may only be authorized to carry out endonuclease activity upon posttranslational modification. The finding here that Pso2 has structure-specific endonuclease activity toward DNA hairpins opens up many fascinating possibilities for potential roles of Pso2 in the ability of yeast and human cells to protect themselves against DNA damage.

In the work reported here, Pso2 activity was analyzed in the context of a closed hairpin with no DNA nicks. Since Mre11/Sae2 hairpin processing is only efficient when a nick is already present (Lengsfeld et al, 2007) and this was not available in the hairpin substrate used, it is not surprising that Pso2 was responsible for repair of ~ 90-95% of fully closed hairpins *in vivo*. Mre11, independent of Sae2, has been shown to have weak hairpin opening activity *in vitro*; and this may account for the small amount of hairpin repair when Pso2 is absent. If these nicks occur at the apex, the resulting DNA end could be repaired like a normal double strand break; however, if nicking takes place distal to the hairpin apex, then Mre11/Sae2 would be free to recognize and process the hairpin intermediate. Under normal DNA damage conditions where hairpin structures are generated, it is likely that some are converted to fully closed hairpins by DNA ligase. In these cases, Pso2 would be expected to function as the dominant hairpin opening activity; however, when a nick remains present, Mre11/Sae2 would be sufficient for hairpin removal. Given the lethality of double

strand break with capped hairpins, it makes sense that cells maintain multiple mechanisms for their repair.

If DNA hairpins are generated during ICL damage and/or repair than one would expect that first, the double strand breaks that occur in a *psb2* null strain would be capped by DNA hairpins and secondly, proteins that are capable of opening DNA hairpins should complement *psb2* null strains in ICL repair.

CHAPTER FOUR

DNA HAIRPINS ARE GENERATED IN RESPONSE TO INTERSTRAND CROSSLINKING EXPOSURE

4.1. Abstract

The eukaryotic mechanism for ICL repair is not thoroughly characterized although a distinguishing feature of this repair pathway is the accumulation of DNA double strand breaks in actively replicating cells subsequent to ICL exposure. The mechanism for how these breaks arise is currently unknown. Pso2 is known to play an important role in processing the double strand breaks generated during repair of ICLs. Given that these breaks are unique from other forms of double strand breaks and that Pso2 has been demonstrated to possess DNA hairpin endonuclease activity, it is possible that DNA hairpin capped ends are the unique feature of these DNA double strand breaks. Yeast Pso2 has three human homologues, SNM1A/B/C. Although SNM1A shares the highest sequence similarity and a shared functional role in ICL repair, Pso2 appears to share several characteristics with Artemis as well, including DNA hairpin opening endonuclease activity. Artemis endonuclease can be constitutively activated by removal of its C-terminal domain, making this construct useful to determine whether hairpin-opening activity can complement a *pso2* null strain in ICL repair. In this study, it was demonstrated that only the β -CASP domain of Artemis, which possesses constitutively active DNA hairpin opening endonuclease activity, could complement a *pso2* deficient strain, while full length Artemis, which possesses only exonuclease activity, was unable to do so. This suggests that it is endonuclease function that suppresses the ICL repair defect in a *pso2* null strain, further supporting the possibility that Pso2 acts on hairpin substrates during

repair of ICL damage. Importantly it was further demonstrated that DNA hairpins structures accumulate in a Pso2 deficient strain but not in wild type *S. cerevisiae*, in response to ICL damage.

4.2. Introduction

Subsequent to ICL exposure, DNA double strand breaks are generated (Lam et al, 2008). These breaks occur far more frequently in actively replicating cells and several models exist to explain how these replication dependant breaks might occur (Dardalhon & Auerbeck, 1995, Magana-Schwencke et al, 1982, McHugh et al, 2000). In the first model endonucleases Mus81 and XPF/ERCC1 (Rad1/Rad10) generate nicks at an ICL lesion. During cell division replication past these nicks, generates double strand breaks. A second model suggests, helicase activity associated with DNA replication induces DNA coiling extruding cruciform. Resolvase recognition and processing of these cruciform structures would generate DNA hairpin capped double strand breaks (McHugh et al, 2000). This second possibility is particularly attractive given that double strand breaks will accumulate even if the candidate endonucleases are deficient. As well, this model would explain why double strand breaks fail to accumulate when the resolvase Mus81 is deficient (Cote & Lewis, 2008, Lobachev et al, 2002). Such a defect is only observed during the S phase of the cell cycle (De Silva et al, 2000, Hanada et al, 2006, Niedernhofer et al, 2004). If DNA hairpin capped double strand breaks are generated during ICL removal, then they must be opened prior to successful ligation and repair of the DNA. Failure to do so may lead to gene

duplication or cell death (Hanada et al, 2006). Pso2 has been shown to open DNA hairpins *in vitro* and *in vivo* and is required for processing double strand breaks generated during ICL exposure. It is therefore likely that if DNA hairpins are generated during ICL damage and repair, Pso2 would be a possible candidate for processing these structures.

Pso2 belongs to the β -CASP family with its closest relatives often referred to as the SNM1 group, including the human homologues SNM1A/B/C. Each of these proteins acts as a 5' exonuclease and all are involved in DNA repair. Pso2 and SNM1A have been thought to be functional homologues given that they are both required for ICL repair and both have their β -CASP domain localized at their C-terminus (Lobachev et al, 2007), (Lenain et al, 2006a, Li et al, 2005, Ma et al, 2002). At this time no yeast homologues for SNM1B (Apollo) and SNM1C (Artemis) have been identified.

SNM1A is involved in the repair of ICL lesions generated through exposure to mitomycin C, however its exact role in this repair pathway is not yet determined (Dronkert et al, 2000a, Ishiai et al, 2004b). Aside from this ICL sensitivity, mammalian cells lacking functional SNM1A are phenotypically normal (Akhter et al, 2004, Dronkert et al, 2000a, Hemphill et al, 2008, Zhang et al, 2002). SNM1B (Apollo) is also required for repair of ICL lesions and a deficiency of this enzyme leads to defects in telomere stability, cell cycle checkpoint, and embryonic development (Akhter et al, 2004, Dronkert et al, 2000a, Hemphill et al, 2008), (Demuth et al, 2004). The role of SNM1A and SNM1B (Apollo) in ICL

repair are not redundant as they are non-epistatic and SNM1A is required for repair of the double strand breaks while SNM1B (Apollo) is required in response to replication fork collapse resulting from exposure to mitomycin C (Ackter 2004) (Ishiai et al, 2004b). Although SNM1C (Artemis) shares the greatest sequence similarity with SNM1B (Apollo), the function of SNM1C (Artemis) is not related to ICL repair but rather processing DNA ends prior to ligation of double strand breaks. It is essential for the repair of coding joints in V(D)J recombination.

When tested for *in vitro* activity together SNM1C (Artemis) and SNM1A have been analyzed for 5' phosphate dependency and hairpin opening endonuclease activity (Demuth et al, 2004, Hejna et al, 2007). Both nucleases require a free 5' phosphate, however, only SNM1C (Artemis) was shown to open DNA hairpins, similar to Pso2 (Ma et al, 2002). SNM1C (Artemis) endonuclease activity requires either post translational modification by DNA-PKcs or complete removal of the inhibitory C-terminal domain (Ma et al, 2002). If hairpin opening activity of Pso2 is required for ICL repair, full length SNM1C (Artemis) would not be able to complement a *pso2* null strain given that there is no DNA-PKcs homologue present in yeast and therefore SNM1C (Artemis) endonuclease activity would be suppressed. The β -CASP domain of SNM1C (Artemis) alone, however, has constitutive DNA hairpin opening activity and therefore may complement the *pso2* Δ ICL sensitivity. In fact this activity was required for repair or ICL damage. SNM1B (Apollo) has yet to be tested for hairpin opening endonuclease function, however, it is possible that either its full length or β -CASP

domain may have endonuclease activity. To determine if endonuclease function of SNM1C (Artemis) β -CASP could rescue the repair defect in a *pso2 Δ* strain, complementation with full length and the β -CASP domain of all three SNM1 nuclease was tested for both hairpin opening and ICL survival *in vivo*. It was further demonstrated here that DNA hairpin structures accumulate in a *pso2* deficient strain. These hairpins do not accumulate in wild type cells unless they are exposed to the ICL inducing agent cisplatin. However, if the cells are allowed to recover, only *pso2 Δ* cells retain DNA hairpins and wild type cells are capable of repairing these hairpin structures.

4.3. Materials & Methods

4.3.1. Plasmid construction and transformation

hSNM1C (Artemis) 1 – 692 and 1 – 359, hSNM1 1 – 1034 and 707 – 1034, and hSNM1B (Apollo) 1 – 352 and 1 – 326 were amplified by PCR and cloned into pAG-ccdB-GAL yeast expression vector using Gateway cloning technology (Invitrogen). The validity of the sequence was verified by DNA sequencing (Mobix, McMaster University). Expression vectors were transformed into a *pso2 Δ* strain to analyze ICL sensitivity and co-transformed with pWL201 to test for hairpin opening activity.

4.3.2. Survival Assay

S. cerevisiae *pso2* null mutants containing hSNM1C (Artemis) 1 – 359 within pYES-DEST52 (invitrogen) were grown in drop out base media containing raffinose as a carbon source overnight at 30°C. Cells were diluted in drop out base media containing galactose as a carbon source with 1 mM cisplatin and grown at 30°C for an additional 2 hrs. Subsequent to resuspension in drop out base media, the cultures were plated out in serial dilution. The number of colonies were counted and compared to a control in the absence of cisplatin to assess the percent survival. The experiment was performed in triplicate.

4.3.3. *In vivo* hairpin opening

S. cerevisiae MYO37 was generously provided by Cliff Weil (Department of Agronomy, Purdue University) and transformed with pWL201 (Ma et al, 2002, Ma et al, 2005, Niewolik et al, 2006) as well as pAG-ccdB-Gal harboring each of the β -CASP enzymes. These cells were grown to saturation at 30°C in drop out base media containing raffinose as a carbon source. Induction of transposase and β -CASP proteins was carried out by the addition of 1% galactose. Cells were allowed to grow at 30°C overnight prior to serial dilution and plating onto media lacking either uracil and histidine or uracil, histidine and adenine. Transposition frequency was determined by dividing the number of *ade*⁺ revertants by the total number of colony forming units.

4.3.4. Two-dimensional analysis of chromosomal DNA

Wild type and *pso2Δ S. cerevisiae* strains were grown in 100 mL of YPD media, overnight at 30°C. Cultures were pelleted prior to resuspending in equal volume of YPD supplemented with a final concentration of 1 mM cisplatin. Cells were incubated with cisplatin at 30°C for 2 hours. Cells were then pelleted and resuspended in fresh YPD and further incubated for 1 hour at 30°C. Final pellets were lysed by resuspending in 2 mL of buffer containing Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris (pH 8) and 1 mM EDTA (pH 8), 3 g glass beads, and 2 mL phenol:chloroform:isoamyl alcohol 25:24:1. Cells were vigorously vortexed for 5 min prior to addition of 2 mL TE buffer, and followed by centrifugation for 5 min at 10 000 g. The aqueous layer was added to 95% ethanol to a final concentration of 70% and centrifuged as before to pellet DNA which was allowed to completely dry prior to resuspension in 200 µL H₂O. In order to simplify further analysis, DNA was incubated at 37°C for 14 days permitting all interstrand crosslinks to convert to intrastrand crosslinks. DNA was loaded onto a 0.5 % TAE agarose gel (12 cm x 14 cm) and run in 1X TAE buffer at 25 V for 6 hours. The lane containing DNA was cut out, rotated 90°, and run in a second dimension at 50 V for 16 hours in buffer containing 40 mM NaOH, and 1 mM EDTA. The gel was neutralized by incubation in H₂O for 30 min followed by two successive washes in 50 mM Tris 7.0. DNA was visualized via incubation in 1:10000 GelStar Nucleic Acid Stain (Lonza).

4.4. Results

4.4.1. Hairpin opening of the β -CASP domain of human SNM1C (Artemis)

In order to determine if SNM1C (Artemis) 1-359, a constitutively active DNA hairpin opening endonuclease domain could complement *pso2* Δ strain in ICL resistance, it must first be verified that this human protein can open DNA hairpins within the yeast cell. The previously described transposon based assay (Chapter 3) was utilized to determine this activity. As shown in Figure 4.1, the β -CASP of SNM1C (Artemis) was able to partially complement at (6 fold above the *pso2* null strain).

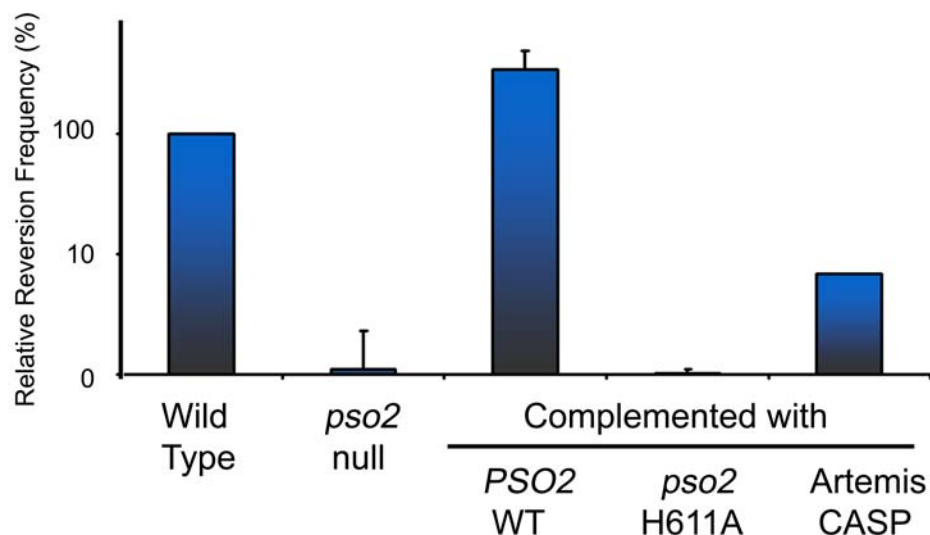
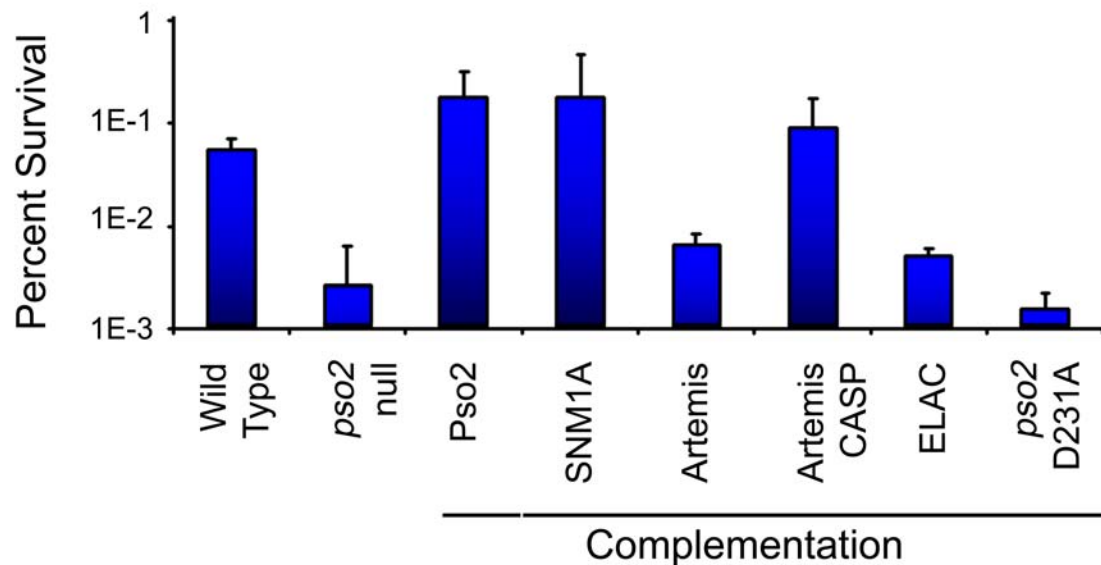


Figure 4.1 SNM1C (Artemis) β -CASP can open DNA hairpins in *S. cerevisiae*. Reversion frequency, normalized to wild type level is plotted for *pso2* null strain complemented with wild type Pso2, *pso2* H611A and SNM1C (Artemis) 1-359, relative to wild type levels. Reversion frequency correlates with ability to repair chromosomal DNA hairpins generated via excision of the Ac transposon from within the *ADE* gene. *Pso2* null strains have decreased repair, which is only restored by complementation with a wild type copy or partially complemented by SNM1C (Artemis) 1-359.

4.4.2. Human SNM1A and the β -CASP domain of SNM1C (Artemis) can restore ICL resistance in a *pso2* null strain

Our data suggests that Pso2 is responsible for opening hairpin structures generated during transposon excision. It is unlikely that the 5'-exonuclease activity of Pso2 would be required for repair of transposon mediated double strand breaks which had already been opened by another endonuclease since Pso2 is not required for repair of double strand breaks generated by IR, HO endonuclease, bleomycin or UV exposure (Yu et al, 2004). Rather it would appear that the structure-specific endonuclease activity of Pso2 is responsible for repair of hairpin structures *in vivo*. If so, the repair defect in a *pso2* deletion strain should be suppressed by an activity that is able to open hairpins. This possibility was tested by complementing a *pso2* Δ strain with the β -CASP domain of human SNM1C (Artemis), which is capable of opening DNA hairpins in *S. cerevisiae*. As shown in Figure 4.2, the β -CASP domain of SNM1C (Artemis) was able to suppress the repair defect in Pso2 deficient cells. SNM1C (Artemis) 1-359 also possesses 5'-exonuclease activity. To rule out the possibility that it is SNM1C (Artemis) exonuclease activity that restores ICL resistance, SNM1C (Artemis) full length, which lacks endonuclease activity while retaining exonuclease activity, was tested for its ability to complement. Full length SNM1C (Artemis) complementation was sensitive to ICL treatment, suggesting that it is SNM1C (Artemis) endonuclease and not its exonuclease function that is responsible for ICL resistance. SNM1A, the closest functional Pso2 homologue

was also able to complement a *Pso2* null strain while the more distinct β -CASP member, ELAC, was unable to do so.



Strain 1	Strain 2	two tailed P value
Pso2 null	Wild type	0.0002
Pso2 null	Complement wild type	0.0201
Pso2 null	Complement SNM1A	0.2014
Pso2 null	Complement Artemis	0.0641
Pso2 null	Complement Artemis CASP	0.0523
Pso2 null	Complement ELAC	0.1871
Pso2 null	Complement H611A	0.5605

Figure 4.2. Cisplatin survival assay with β -CASP complements. Survival after 2-hour incubation with increasing concentration of cisplatin for complementation with β -CASP members. Only *Pso2*, SNM1A, and SNM1C (Artemis) 1-359 can restore the repair defect in the *pso2* null strain.

4.4.3. DNA hairpins accumulate subsequent to ICL damage

Given the ability of Pso2 to open DNA hairpin structures *in vitro* and *in vivo*, and its specialized role in ICL repair, it was of interest to determine if similar structures were generated during ICL exposure and whether Pso2 was able to mediate their repair. Two-dimensional (native and denaturing) analysis was performed to monitor the presence of DNA hairpins. DNA from wild type cells migrates along a single curved diagonal indicating dsDNA while DNA from *pso2Δ* has an additional DNA species migrating slower than the diagonal indicating DNA hairpin structures formed in response to ICL damage and/or repair (Figure 5.1, top panels). These DNA hairpins are present even in the absence of an ICL agent suggesting that hairpin structures are present within the cell and processed by Pso2. It is unclear whether these hairpins are formed from extrusions of the DNA or if they are present at DNA double strand breaks. Given that *pso2* null strains are phenotypically normal it is likely that these hairpins are the result of cruciform structure converted to DNA capped ends during DNA extraction and fragmentation. Following cisplatin exposure DNA hairpin species are generated indicating that hairpins are in fact generated in response to ICL inducing agents (Figure 5.1, bottom panels).

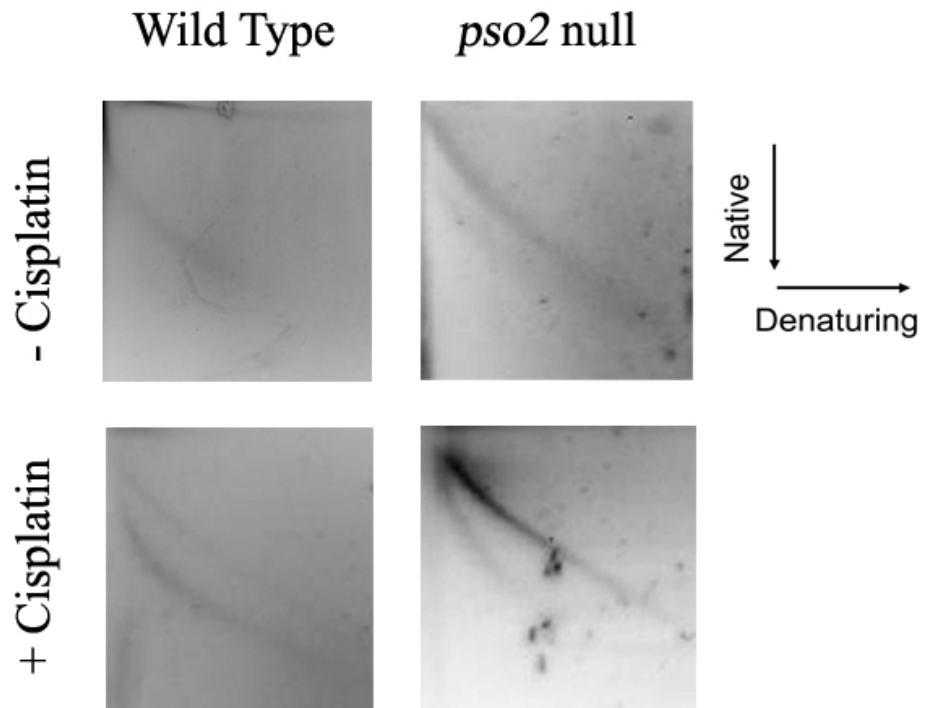


Figure 4.3 Analysis of *S. cerevisiae* extracted DNA reveals DNA hairpins are generated subsequent to ICL damage. Two-dimensional (native followed by denaturing) analysis was performed on chromosomal DNA extracted from *S. cerevisiae* treated with and without cisplatin. The lower 'arc' present in the upper right panel corresponds to DNA with hairpin-like mobility. This arc was present in the absence of Pso2 and was generated in wild type DNA only after treatment with cisplatin.

4.4.4. *Pso2 is required for repair of DNA hairpins generated by ICL repair*

To determine whether Pso2 is required for processing of DNA structures generated following ICL exposure, the cells were allowed to recover following treatment. DNA hairpins were observed directly after treatment in both wild type and *pso2Δ* cell lines (Figure 4.3) however, these DNA hairpins were repaired in wild type cells after recovery (Figure 4.4). These structures represent true DNA hairpins as they are resolved by treatment with mung bean nuclease, which is unable to cleave DNA interstrand cross-links (Barber et al, 2005, Henriques & Moustacchi, 1980, Lam et al, 2008). Therefore, Pso2 is able to open chromosomal DNA hairpins generated either through transposon excision or exposure to ICL damage.

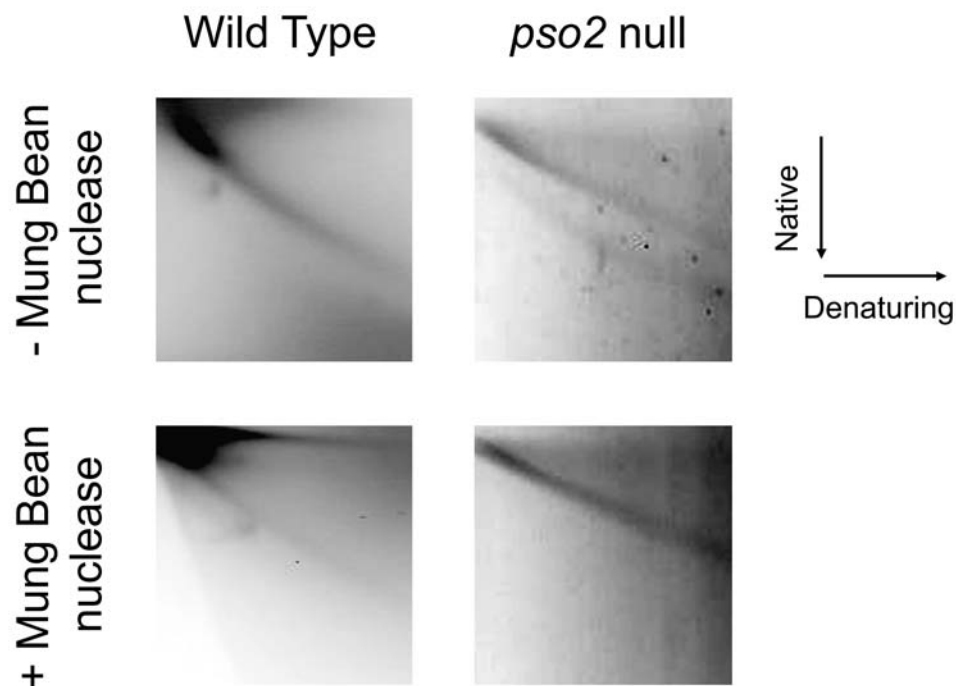


Figure 4.4 Analysis of Pso2 activity toward DNA intermediates generated in response to ICL exposure. Two-dimensional (native followed by denaturing) analysis was performed on chromosomal DNA extracted from *S. cerevisiae* treated with cisplatin. The lower ‘arc’ present in the upper right panel corresponds to DNA with hairpin-like mobility. This arc was only present in the absence of Pso2 and was able to be resolved by exogenous addition of hairpin opening activity (mung bean nuclease).

4.5. Discussion

Pso2 possesses a unique function permitting it to process some intermediate during ICL repair. Given that Pso2 has been shown to open DNA hairpins *in vitro* and *in vivo* (Chapter 3), it follows that DNA hairpins may represent the structure Pso2 acts upon during repair of ICL damage. All of the human Pso2 homologues possess 5' exonuclease activity, yet only full length SNM1A has been shown to complement a *pso2* null strain (Lucchini et al, 2001, Patrick & Turchi, 1999). This suggests that 5' exonuclease activity is not the role Pso2 plays in ICL repair. The presence of several redundant exonucleases in yeast further supports this hypothesis. Although full length SNM1C (Artemis) possesses DNA exonuclease, it was unable to complement a *pso2* null strain. SNM1C (Artemis) endonuclease function is regulated through phosphorylation of its C-terminal domain by DNA-PK to relieve inhibition of this endonuclease activity. Given that there is no DNA-PK yeast homologue it is not surprising that full length SNM1C (Artemis) endonuclease activity would remain suppressed. Removal of the C-terminal domain of SNM1C (Artemis) results in constitutively active DNA hairpin opening endonuclease activity *in vitro* (Hazrati et al, 2008b). Here it was shown that this constitutively active SNM1C (Artemis) domain is able to complement a *pso2* deficient strain in a DNA hairpin opening assay as well as ICL repair. Given that full length SNM1C (Artemis) is unable to complement a *pso2* null strain, it would appear that SNM1C (Artemis) endonuclease activity is functioning in ICL repair. Furthermore, it was demonstrated that DNA hairpin are

generated subsequent to ICL treatment and that Pso2 is required to process these hairpins.

4.5.1. Shared nuclease activities within the β -CASP family

β -CASP proteins [CPSF-73, ELAC2, Pso2 (SNM1A), Apollo (SNM1B) and Artemis (SNM1C)] are characterized by a large insertion (CASP domain) into an otherwise canonical metallo- β -lactamase domain (Niewolik et al, 2006). The structure of CPSF-73, determined by x-ray crystallography, revealed that the CASP domain forms a lid covering the metallo- β -lactamase active site. Residues from within the CASP domain have been shown to play a direct role in both substrate recognition and catalysis (Callebaut et al, 2002). Unfortunately, the crystal structure did not contain bound substrate and therefore details regarding this aspect of β -CASP function are still unclear.

Although the biological function of β -CASP proteins is diverse, spanning telomere maintenance, RNA processing, and both DNA double strand break and ICL repair, they all function as nucleases. Work presented here demonstrated that Pso2 acts as an efficient 5'-exonuclease, independent of DNA structure. Interestingly, this activity was found to be absolutely dependent on the presence of a 5'-phosphate upstream of the cleaved phosphodiester bond. CPSF-73, SNM1A, SNM1C (Artemis) and SNM1B (Apollo) also function as 5'-exonucleases, albeit with more defined preferences toward distinct nucleotide structures (Li & Moses, 2003, Pannicke et al, 2004, Ryan et al, 2004). This shared similarity of 5'-exonuclease activity extends to a 5'-phosphate

dependence as well. Only human SNM1A and SNM1C (Artemis) have been tested in this regard, but both show the same dependence as Pso2 for a free 5'phosphate (Hejna et al, 2007, Lenain et al, 2006b, Ma et al, 2002, Ryan et al, 2004). Such dependence is rare amongst nucleases and appears to be one of the defining features of the β -CASP active site.

Why a 5'-exonuclease would require a free 5'phosphate for catalytic activity is puzzling. The fact that β -CASP proteins maintain this feature from yeast to humans suggests that it is conserved for an important functional reason. In the case of Pso2, the presence of a free phosphate is not important for binding substrate. This implies a more direct role for this phosphate in catalysis. One possible explanation for the conserved dependence of β -CASP exonuclease activity on a 5'-phosphate may be related to the ability of β -CASP proteins to function as structure-specific endonucleases. Pso2, CPSF-73, Elac2 and SNM1C (Artemis) cleave their endonuclease substrates at or close to single-double strand junctions (Hejna et al, 2007, Ma et al, 2002). In all cases, cleavage is only observed when the substrate contains an unpaired base on the 5' side of the cleaved bond (Figure 4.3). The presence of an unstacked base at this position could permit β -CASP proteins to engage a 5'-phosphate during endonucleolytic cleavage in much the same way as they bind a free 5'-phosphate during exonuclease cleavage events. Such a relationship would account for the observed dependencies for substrate specificities in both endo and exonuclease functions. Further structural studies of β -CASP proteins in complex with their

substrates will be required to fully understand the relationship between exo and endonuclease catalytic mechanisms.

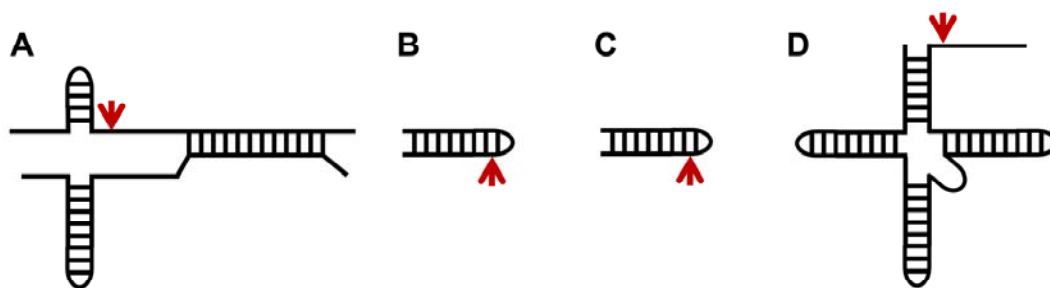


Figure 4.5 Endonuclease Substrates of β -CASP proteins. **(A)** CPSF possesses endonuclease activity towards RNA (Ryan K *et al.*, 2004; Yang *et al.*, 2009). **(B)** Artemis nicks DNA hairpin structures at the ss- to ds-DNA junction, 2 nucleotides from the hairpin apex on the 3' side (Ma *et al.*, 2002). **(C)** Pso2 has the same structure specific endonuclease activity observed for human SNM1C (Artemis). **(D)** ELAC2 has not been shown to open DNA hairpins; nonetheless, cleaves the first unpaired nucleotide 3' to the tRNA acceptor stem (Takaku *et al.*, 2003). DNA hairpins closely resemble this structure as nucleotides proximal to the apex would not be paired. Both SNM1A and SNM1B (Apollo) have domains in addition to the β -CASP domain that may be regulatory domains. Perhaps modification or removal of these domains may lead to endonuclease activity.

4.5.2. Pso2 endonuclease activity in ICL repair

The mechanism of ICL repair in eukaryotes is still poorly understood. Although studies have identified key proteins in ICL repair, it is not yet clear how these proteins function to generate DNA intermediates suitable for repair. Pso2 (SNM1A) represents one of only a small number of proteins that function predominantly in ICL repair (Ma et al, 2002, Takaku et al, 2003). The finding that Pso2 is able to open DNA hairpin structures *in vitro* and *in vivo* suggests that this activity may be important for processing related DNA intermediates generated directly or indirectly in response to ICL damage.

How might DNA hairpins be generated during ICL damage and/or repair? Several possibilities exist. Introduction of a DNA interstrand crosslink creates a physical barrier to DNA replication, causing stalling, fork collapse and formation of double strand breaks (Brendel et al, 2003a). In support of this idea, double strand breaks are found to accumulate to a greater extent in actively versus non-actively replicating cells following exposure to ICL-inducing agents (Michel et al, 1997). Brendel *et al* (2003) proposed a model whereby movement of the replication fork toward an ICL lesion would cause cruciform extrusion (Figure 4.6). These structures could be generated on both sides of an ICL lesion due to topoisomerase I activity functioning ahead of the replication fork. Similar to cruciforms formed at long inverted repeats in yeast (McHugh et al, 2000), cruciforms generated by replication toward an ICL would be converted into various sized hairpin-capped double stranded breaks (Figure 4.6) (Lobachev et

al, 2002). These substrates may in turn serve as intermediates for MRX and/or Pso2 endonuclease activities. Consistent with this hypothesis, deletion of *PSO2* has been shown to result in accumulation of double strand breaks (Cote & Lewis, 2008). As such, it is thought that Pso2 functions subsequent to DSB formation in ICL repair. The biochemical and genetic analysis of Pso2 presented here, is consistent with at least a subset of these double strand breaks being capped by hairpin structures.

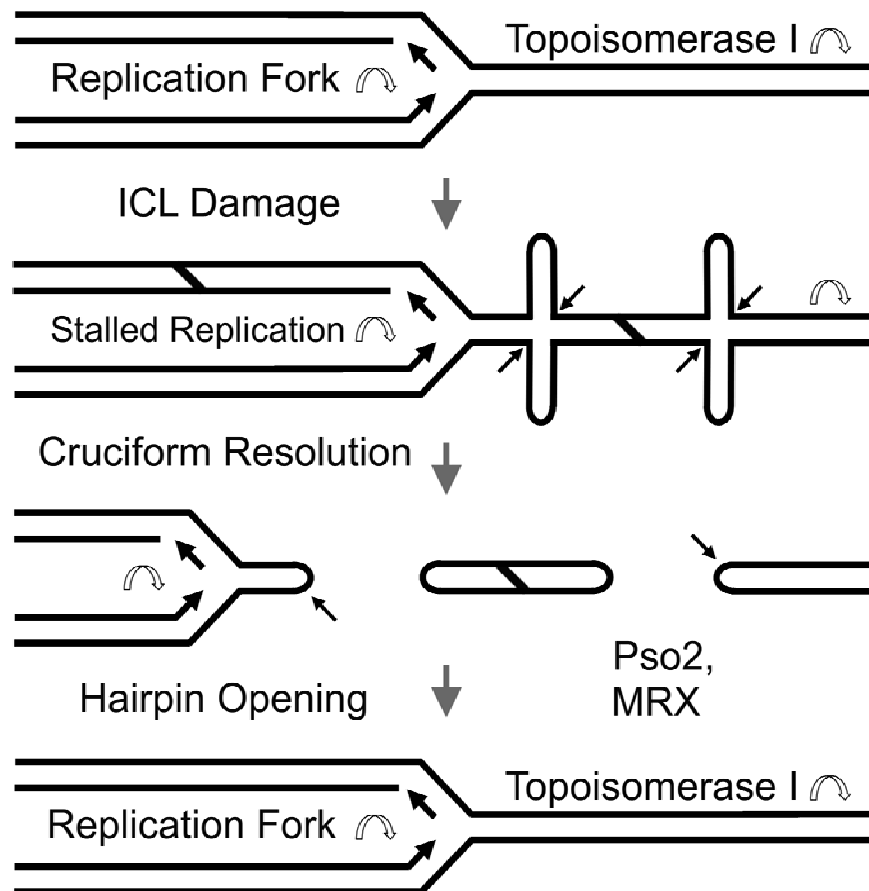


Figure 4.6 Model for generation and repair of hairpins during ICL damage. As the replication fork moves toward an ICL lesion helicase causes cruciform extrusion between the fork and ICL. Topoisomerase I activity ahead of the ICL also may create cruciform structures. Cruciform structures are resolved into hairpin capped double strand breaks (Lobachev et al, 2002; Cote and Lewis, 2008). Pso2 and/or the MRX/Sae2 complex process hairpin structures for subsequent end joining repair.

A second possibility for how Pso2 might utilize its structure-specific endonuclease function in ICL would be repair, not of DNA hairpins, but rather of 'hairpin-like' structures. The crystal structures of a fully paired hairpin and of a cisplatin-generated interstrand crosslink have been determined (Li & Moses, 2003). Comparison of these DNA structures reveals several intriguing similarities. Introduction of a crosslink between G bases from opposing strands of the duplex DNA causes C bases on each of the 3' sides of the ICL to be completely flipped out of the duplex (Figure 4.7). This results in the presence of a free phosphate that closely resembles the structure formed at the apex of a fully paired DNA hairpin. Thus, Pso2 may be able to recognize such a substrate and cleave the hairpin-like structure on the 3' side of an ICL lesion. Even if a double strand break were not generated close to an ICL, the distortion created by ICL damage may be sufficiently similar to a DNA hairpin for Pso2 to recognize and cleave the lesion. Further analysis is required to investigate if the DNA hairpin opening activity of Pso2 reported here is involved in ICL repair and if so, at what point in the repair process such an intermediate would be encountered.

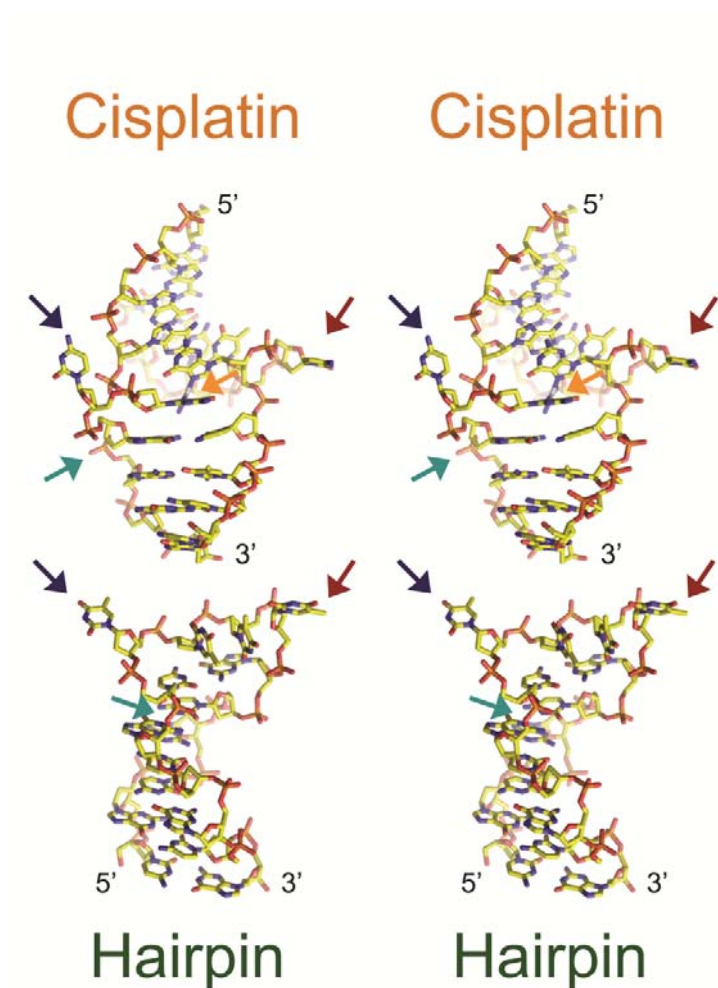


Figure 4.7 Structural comparison of a DNA hairpin and cisplatin DNA lesion. A stereo view of the structures of a DNA hairpin (PDB accession code 1D16) and cisplatin adduct (PDB accession code 1A2E) are shown. Orange arrow, cisplatin mediated interstrand crosslink located between opposing G bases; dark blue arrows, 3' bases flipped out of helix; red arrows, 5' bases flipped out of helix; cyan arrows, analogous scissile phosphates for Pso2 endonucleolytic cleavage located 2 bases from apex.

4.6. Conclusion

In this study, it was demonstrated that a constitutively active endonuclease domain of SNM1C (Artemis) was able to open DNA hairpins in *S. cerevisiae* as well as complement a *pso2* deficient strain in ICL repair. Full length SNM1C (Artemis) and SNM1B (Apollo) both possess 5' exonuclease activity and were unable to complement this strain suggesting that it is DNA hairpin endonuclease and not 5' exonuclease activity that restores the *pso2* defect. The finding that Pso2 displays hairpin-opening activity toward intermediates generated in response to ICL exposure is particularly interesting and suggest that the human homologue of Pso2 (SNM1A) may also be involved in processing similar DNA intermediates. Given that unregulated nuclease activity is associated with cell toxicity, SNM1A endonuclease activity would be expected to be highly regulated in mammals. Therefore, like its orthologue SNM1C (Artemis), SNM1A may only be authorized to carry out endonuclease activity upon posttranslational modification. The finding here that Pso2 has structure-specific endonuclease activity toward DNA hairpins opens up many fascinating possibilities for potential roles of Pso2 in the ability of yeast and human cells to protect themselves against DNA damage.

CHAPTER FIVE

CONCLUSION AND DISCUSSION

5.1. Overall Conclusion and Relevance

The work reported here demonstrates that Pso2 is a processive 5' exonuclease lacking structure specificity aside from an unusual requirement for a 5' phosphate. Interestingly, occupying the phosphate interacting region of Pso2 with phosphite inhibits 5' exonuclease activity despite this region being important for association with DNA suggesting that a 5' phosphate is required indirectly for catalysis. A 5' phosphate requirement, although atypical for nuclease enzymes, is common amongst the SNM1 family in which three of the four members, SNM1A, SNM1C (Artemis), and Pso2 have been shown to require this 5' phosphate for activity (Henja *et al*, 2007; Ma *et al*, 2002). The fourth member SNM1B (Apollo), has not been analyzed in this regard. Despite the growing evidence for an important role in catalytic function, a precise role for this phosphate requirement is currently unavailable. One possibility is that this phosphate may prevent unwarranted DNA degradation. It is also possible that this phosphate requirement may be related to endonuclease function. SNM1C (Artemis) cleaves DNA hairpins two nucleotides from the apex at the 3' side. A crystal structure of a DNA hairpin (Chapter 4, Figure 4.7) reveals that the phosphodiester backbone at the unpaired nucleotides of the DNA hairpin end resembles a free 5' phosphate. Accordingly, SNM1C (Artemis) endonuclease cleavage occurs at the subsequent phosphodiester bond perhaps mimicking its substrate requirement for 5' exonuclease activity. Work reported here shows that Pso2 efficiently cleaves DNA hairpins, two nucleotides from the apex in a manner

indistinguishable from SNM1C (Artemis). This activity is structure-specific as Pso2 is unable to cleave ss, ds- or ss-to-ds-DNA junctions.

The knowledge that a 5' phosphate is required for exonuclease activity and that occupying this interacting region abolished activity could be useful in future structure based drug design initiatives. Both SNM1C (Artemis) and SNM1A represent ideal targets given their roles in DNA repair. Agents currently used in cancer treatment often damage DNA, however, resistance emerges due to upregulation of repair factors (Chattopadhyaya et al, 1990, Coste et al, 1999b). Abolishing these repair pathways in combination with DNA damaging treatments has great potential for combating tumorigenesis. Furthermore the Pso2 human homologue, SNM1A, displays no phenotypical abnormalities when depleted from mammalian cell lines suggesting inhibition directly towards SNM1A would have limited toxicity (Plummer, 2010).

Pso2 was analyzed for its ability to open DNA hairpins generated within *S. cerevisiae*. These hairpin capped double strand breaks were generated via Ac/Ds transposon excision where the removal of this mobile DNA element leaves DNA hairpin capped ends on the host DNA (Weil and Kunze, 2000; Zhou *et al*, 2004). Such structures must be opened in order to carry out successful ligation. Consistent with the *in vitro* hairpin opening activity of Pso2, DNA repair was reduced in a *pso2* deficient strain when hairpin structures were generated. Given that Pso2 is not required for repair of traditional double strand breaks and that these breaks differed only by the presence of a DNA hairpin indicates that Pso2

was required to process these hairpin structures (Henriques *et al*, 1980; Barber *et al*, 2005; Lam *et al*, 2008). Hairpins generated by Ac/Ds transposition resemble hairpins generated by RAG1/2 during V(D)J recombination as they are both generated a transesterification reaction involving nucleophilic attack of a free 3'-OH from one strand on the phosphodiester bond of the immediately juxtaposed position of the opposing strand (Akhter *et al*, 2004, Dronkert *et al*, 2000a, Hemphill *et al*, 2008, Bhasin *et al*, 1999, Kennedy *et al*, 1998). It is interesting that hairpin-processing factors such as MRX/N do not act upon hairpins generated by Ac/Ds transposase or RAG1/2 generated hairpins. It has been shown that MRX and Pso2 may act on similar but slightly distinct substrates. Pso2 has no sensitivity towards ionizing radiation yet it further sensitizes an *mre11* deficient strain in a double mutant strain (Lam *et al*, 2008). Furthermore, Mre11 shows no defect in repair of cisplatin induced damage, however, double mutants are more sensitive to cisplatin than the *pso2* null strain alone (Lam *et al*, 2008). Taken together these differences in DNA structure specificity for Mre11 and Pso2 are not currently known. Results suggest, Mre11 hairpin opening endonuclease activity is reduced as the size of the hairpin loop is decreased. Since hairpins generated by transposon excision and V(D)J recombination are fully paired the hairpin processing specificity of Mre11 and Pso2 may be determined by the length of the DNA hairpin, where MRX requires larger hairpins and Pso2/ SNM1C (Artemis) utilizing smaller hairpin structures (Paull and Gellert 1998).

Given the specialized role of Pso2 in ICL repair and its ability to open hairpin structures *in vivo* and *in vitro* it was of interest to determine whether Pso2 endonuclease activity was required for ICL repair. A DNA hairpin opening endonuclease domain of SNM1C (Artemis) was analyzed for its ability to complement a *pso2* null strain in ICL repair. This SNM1C (Artemis) domain was able to restore ICL sensitivity while full length SNM1C (Artemis) in which endonuclease function is suppressed, was unable to do so. The human Pso2 homologue, SNM1A, was also able to complement *pso2* Δ suggesting that all three proteins are capable of acting on similar substrates. This raises the possibility that mammalian ICL removal shares similarities with yeast repair with respect to processing of hairpin structures.

The role of Pso2 endonuclease activity in ICL repair is not currently established. In light of the work presented here at least two potential possibilities exist; Pso2 may act upon hairpins generated during repair of ICL damage or perhaps on hairpin like substrates directly formed as a consequence of ICL damage. A model has been proposed in the literature in which replication machinery approaching a ICL lesion would induce strain upstream of the ICL damage while topoisomerase I acting downstream of the damage would generate torsion on the DNA (Brendel, et al 2008). The net result of this unresolved torsion would be DNA extrusions generating cruciform structures (Chapter 4, Figure 4.6). Cruciforms are recognized by resolvases as Holliday junction-like structures generating DNA double strand breaks with DNA hairpin

ends (Lobachev et al, 2002; Cote and Lewis, 2008). In fact, several complexes involved in Holliday junction resolution are also required in ICL repair such as Mus81 and SLX4 (van Gent et al, 1996). Such ends are unable to be ligated until processed to either remove or open the DNA hairpin ends. DNA double strand breaks are not repaired in a *pso2* null strain subsequent to ICL exposure. Importantly, this does not occur with other forms of DNA damage (Henriques *et al*, 1980; Li *et al*, 2003; Barber *et al*, 2005; Lam *et al*, 2008). If double strand breaks generated in ICL damage contain DNA hairpins, this would account for the accumulation of these breaks in the absence of Pso2 even though all other members of DNA DSB repair pathways are present. Furthermore, it is possible that Pso2 may act directly on DNA hairpin-like substrates generated through ICL damage. Structurally, a DNA crosslink may resemble a DNA hairpin in that nucleotides are unpaired near and at the ICL lesion. In fact, cisplatin generated ICL damage significantly resembles a DNA hairpin in that the unpaired nucleotides are forced out of the helix (Coste *et al*, 1999). Pso2 may recognize this DNA structure and unhook the ICL directly. In support of this possibility, incision at ICL damage occurs even in the absence of the NER enzymes proposed to perform this unhooking step (Hanada et al, 2006, McPherson et al, 2004, Yamamoto et al, 2011). Furthermore, a study by De Silva et al (2000) and Niedernhofer et al (2004) demonstrates that endonuclease cleavage occurs on the lagging strand and given that ERCC1/XPF cleave 5' to the damage and Mus81 cleaves nicked substrates, an additional endonuclease would be required

to cleave 3' to the ICL (Raschle et al, 2008). XPG (Rad2), the 3' endonuclease involved in NER, does not appear to have an important role in ICL repair (Bardwell et al, 1994, Gaillard et al, 2003). XPG acts as a single strand endonuclease requiring helical unwinding of the DNA prior to incision during NER. Given that XPG cleaves only six nucleotides from pyrimidine dimers it is possible that helicases involved in NER or cellular replication are unable to approach the ICL in close enough proximity to generate appropriate substrates for XPG. Pso2 however cleaves hairpin structures 3' to the apex suggesting that if acting on ICL substrates, it would cleave 3' to the lesion. In the absence of Pso2, nicking would then occur on the lagging strand 5' but not 3' to the ICL. In this way, a DSB would be generated at the lagging strand. However, without full unhooking of the DNA lesion, the crosslinked sequence could not be removed to repair the DNA. The broken end would be unable to restart the replication fork and therefore these breaks would accumulate as is observed in the *pso2* null strain. This would explain the requirement of Pso2 endonuclease activity in ICL repair as would recognize the ICL damage as a hairpin-like substrate and aid in the unhooking of the damage.

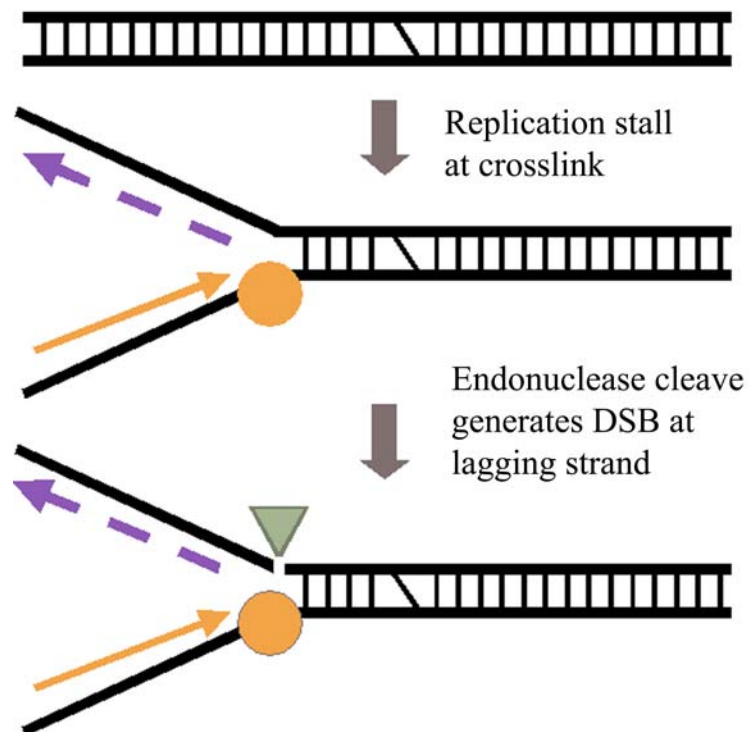


Figure 5.1 Model for double strand break accumulation in the absence of Pso2. Nicking by endonuclease activity occurs 5' of the interstrand crosslink (green triangle) on the lagging strand generating a double strand break. If Pso2 is the enzyme responsible for unhooking the interstrand crosslink at the 3' side, than in the absence of pso2, removal of the damage would not occur. In this case, the double strand break would be unable to restart the replication fork.

To identify whether hairpins are generated in response to ICL damage, two-dimensional agarose analysis was performed. This work suggests that DNA hairpins are generated in response to ICL damage and that these hairpin structures persist in the absence of Pso2. This 2D analysis of chromosomal DNA from cells treated with cisplatin further supports the suggestion that Pso2 may function in ICL repair at least in part by processing DNA hairpin structures. These findings add significantly to the current understanding of ICL repair and raise further questions as to the mechanism of how these unique hairpin structures are generated.

5.2. Future Directions

Findings reported here demonstrate that Pso2 may be acting on DNA hairpin ends during ICL repair. It has not been established how or when these hairpins would be generated. Furthermore, the possibility of Pso2 acting as an endonuclease at ICL lesions has not been ruled out. To address the first question, Pso2 must be tested against a substrate containing an internal ICL. The substrate analyzed in chapter 3 (Figure 3.1) was used to test translesional exonuclease activity. In this assay the lesion was placed five nucleotides from the 3' label. Nucleotides at the end of this substrate were unable to base pair at the temperature in which the experiment was conducted and therefore this substrate did not represent a true ICL substrate that Pso2 may encounter *in vivo*. A longer substrate containing an internal ICL must be analyzed to determine if Pso2 is capable of endonuclease cleavage of this substrate. It would also be

important to test this substrate having a 5' nick similar to what is expected to occur *in vivo* through the action of Rad1/Rad10. To address how and when DNA hairpins may be generated, the described 2D gel analyses may be conducted with strains lacking possible resolvase candidates involved in ICL repair such as *mus81* as a single mutant or in combination with *pso2*. If DNA hairpins are not generated in these strains this would support the model in which cruciform structures are cleaved subsequent to ICL damage. Furthermore, it would be expected that deletion of *pso2* would not display further sensitivity towards ICL damage in a *mus81* null background.

S. cerevisiae is a useful model system for understanding human DNA repair pathways given the similarity of repair mechanism and simplicity of gene manipulation in this lower organism. The findings reported here suggest that human SNM1A may act on a similar substrate as yeast Pso2 since SNM1A was able to complement a *pso2* null strain following ICL damage. To further verify this possibility, SNM1A should be analyzed directly *in vitro* for its nuclease function. Currently, very little biochemical work has been performed with SNM1A due to the difficulty associated with its expression and purification. Biochemical studies of SNM1A have been reported. In these studies DNA hairpin opening endonuclease activity was not detected, however, like SNM1C (Artemis) this activity may be tightly regulated. To identify if SNM1A possesses regulated endonuclease activity, various domains can be analyzed individually. Similar to SNM1C (Artemis) the β -CASP domain of SNM1A may display constitutive

endonuclease activity on its own. Additionally, purified SNM1A may be treated with lysate from human cell lines pre-treated with ICL damaging agents to assess whether post-translational modification is involved in regulating potential hairpin opening activity.

This research of the *S. cerevisiae* ICL repair pathway has led to the discovery of a unique hairpin intermediate and elucidates the requirement of the specialized nuclease Pso2. Further similar studies must now be conducted in mammalian cell lines to determine if a similar mechanism is occurring in higher eukaryotes.

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